VOLUME 2 DECEMBER 1956 NUMBER 7

Canadian Journal of Microbiology

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OTTAWA CANADA

CANADIAN JOURNAL OF MICROBIOLOGY

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Canadian
Journal of Microbiology

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

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ON THE MECHANISM OF THE AEROBIC OXIDATION OF D-XYLOSE AND OF D-RIBOSE-5-PHOSPHATE BY CELL-FREE EXTRACTS OF PSEUDOMONAS HYDROPHILA[1]

BY B. A. STONE[2] AND R. M. HOCHSTER[3]

Abstract

Under aerobic conditions, cell-free extracts of *Pseudomonas hydrophila*, grown on D-xylose, oxidize D-xylose and D-ribose-5-phosphate by identical terminal pathways. Diphosphopyridine nucleotide-linked oxidation of either substrate is much more vigorous than the equivalent triphosphopyridine nucleotide-linked system. Sedoheptulose-7-phosphate is an intermediate in the presence of either substrate or cofactor. D-Glyceraldehyde-3-phosphate is oxidized in the presence of diphosphopyridine nucleotide with the concomitant uptake of the same amount of oxygen as with xylose or ribose-5-phosphate. Phosphoglycerate and phosphoenol pyruvate are rapidly converted to pyruvate, which, itself, disappears by a pathway not involving further oxygen uptake. Glyceraldehyde phosphate dehydrogenase is shown to be diphosphopyridine nucleotide specific and iodoacetate sensitive whereas glucose-6-phosphate and 6-phosphogluconate dehydrogenases are triphosphopyridine nucleotide specific and iodoacetate insensitive, both in fresh extracts and in ammonium sulphate fractions.

The over-all oxidation is shown to proceed via D-glyceraldehyde-3-phosphate when diphosphopyridine nucleotide is used as coenzyme and most probably via the reactions of the pentose cycle in the presence of triphosphopyridine nucleotide. It is suggested that in the intact cell both processes occur simultaneously and compete with one another.

Introduction

The direct oxidation of D-xylose to D-xylonic acid was first shown by Bertrand (5, 6) in *Acetobacter xylinum*, by Lockwood and Nelson (41) in pseudomonads, by Hayasida (24) and by Angeletti and Cerutti (1) in molds. Xylonic acid accumulated in the media in which most of these organisms were grown and was slowly utilized by some species. It is known also that liver glucose dehydrogenase (22, 50) will catalyze the oxidation of xylose to a considerable extent while mold glucose oxidase (aerodehydrogenase) (33) has only very slight activity with respect to xylose. Wainio (53) found that liver

[1]*Manuscript received July 16, 1956.*
Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, Canada. Issued as N.R.C. No. 4092.
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[The October number of this Journal (Can. J. Microbiol., 2 : 519-622. 1956) was issued November 8, 1956.]

preparations oxidize D-xylose in the presence of DPN,[4] TPN, and methylene blue. DeLey (12) showed that D-xylose is oxidized after a lag period whereas D-xylonate was not oxidized at all by whole glucose-grown cells of *Aerobacter cloacae*. A direct non-phosphorylative pathway for the aerobic oxidation of xylose has not yet proved significant in the metabolism of xylose by micro-organisms and tissues.

In previous publications from this laboratory (26, 27, 28) the anaerobic conversion of xylose to phosphorylated pentose, heptulose, hexose, and triose intermediates was described for cell-free extracts of *Pseudomonas hydrophila*. It was also shown how xylose enters the reactions of the pentose cycle (11, 21) and that ribose-5-phosphate is one of the intermediates in xylose degradation, an observation first reported by Lampen (39) for *L. pentosus*. The complete scheme for the anaerobic conversion of xylose to triose phosphate has been formulated for *P. hydrophila* (28) following recent demonstrations and purifications of xylulose kinase (27, 48, 51) and phosphoketopentoepimerase (51) and the finding that xylulose-5-phosphate rather than ribulose-5-phosphate is the donor substrate for transketolase (49, 30).

Most work in this field has been done with purified and in some cases with crystalline enzymes and little is known about the over-all aerobic oxidation pathway for xylose and for R-5-P. Dickens and Glock (15) found that R-5-P oxidation was completely specific for TPN by ammonium sulphate fractions from clear liver extracts. Sable (46) indicated that both DPN and TPN were reduced by R-5-P in the presence of yeast and liver protein fractions. Axelrod *et al.* (3) also noted some activity with DPN in ammonium sulphate fractions from plant extracts. No such data are available on xylose oxidation.

This paper reports the results of aerobic studies in extracts of *P. hydrophila* which show that xylose oxidation proceeds via a phosphorylative route in xylose-grown cells rather than by direct oxidation, that R-5-P is oxidized by the same route, that sedoheptulose phosphate is an intermediate in the system, and that two distinct oxidative routes function in *P. hydrophila* for the oxidation of xylose and of R-5-P, one being dependent on DPN, the other on TPN.

Methods

Materials

The following substances were commercial preparations: ADP, ATP, DPN, TPN, and CoA, Pabst Brewing Co.; G-6-P, HDP, F-6-P, PGA, and GSH, Schwarz Laboratories; FAD and Tris, Sigma Chemical Co.; glycylglycine and cytochrome *c*, General Biochemicals, Inc.; PEP, California Foundation for Biochemical Research; D-xylose, R-5-P, ThPP, cysteine.HCl,

[4]*The following abbreviations are used: ADP, adenosine diphosphate; ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; FAD, flavin adenine dinucleotide; CoA, coenzyme A; Xu-5-P,* D-*xylulose-5-phosphate; Ru-5-P,* D-*ribulose-5-phosphate; R-5-P,* D-*ribose-5-phosphate; SH-7-P, sedoheptulose-7-phosphate; G-6-P,* D-*glucose-6-phosphate; F-6-P,* D-*fructose-6-phosphate; HDP,* D-*fructose-1,6-diphosphate; 6-PG, 6-phosphogluconate; GAP,* D-*glyceraldehyde-3-phosphate; PGA, phosphoglyceric acid; PEP, phosphoenol pyruvate; GSH, reduced glutathione; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; IOA, iodoacetate; ThPP, thiamine pyrophosphate.*

alcohol dehydrogenase, lactic dehydrogenase (2× crystallized), aldolase (2× crystallized), and glyceraldehyde phosphate dehydrogenase (2× crystallized), Nutritional Biochemicals Corp.

Phosphoglyceric kinase was prepared from pea seed extract by the method of Axelrod and Bandurski (2) and 6-phosphogluconate by a modification of the method of Seegmiller and Horecker (47). D-Glyceraldehyde-3-phosphate was obtained through the courtesy of Dr. C. E. Ballou in the form of the dimethyl acetal and converted to the free acid by the method of Ballou and Fischer (4). Samples of sedoheptulosan monohydrate were kindly donated by Drs. N. K. Richtmyer and J. K. N. Jones. Analytical work with this material was based on calculations of the sedoheptulose content following prior treatment with acid (56). As an accurate analytical standard a sample of sedoheptulose-*o*-nitrophenylhydrazone kindly supplied by Dr. A. C. Neish was used following hydrazine removal (43). Excellent agreement was obtained between values arrived at by the two methods. α-Lipoic acid was the gift of Dr. I. C. Gunsalus.

Analytical Methods

Pentose and heptulose, when present in the same solution, were determined by the method of Horecker, Smyrniotis, and Klenow (32). Their concentrations were calculated by solution of the simultaneous equations given by these authors. Heptulose was also estimated after separation and elution from chromatographic strips by the method of Dische (16) using "Procedure 2" with heating after acetic acid addition. GAP was estimated by the method previously described (28). PGA estimation was based on the formation of the hydroxamic acid of 1,3-diphosphoglyceric acid in the presence of ATP, phosphoglyceric kinase, and hydroxylamine (2). The reaction mixture contained the following components (in micromoles) in a total volume of 3 ml.: hydroxylamine (pH 7.4) 100; $MgCl_2$ 3; ATP 6; PGA 0.1–2.0; and excess kinase. The samples were incubated for 90 min. at 30° C. and the reaction was stopped by addition of 5 ml. of Lipmann and Tuttle's (40) $FeCl_3$–TCA reagent. A linear relationship was obtained for the limits of PGA concentration mentioned above. Readings were taken at 540 mμ. Pyruvate was measured by the double extraction procedure of Friedemann and Haugen (18) using toluene as solvent. Control experiments established that ketopentoses, ketohexose, and ketoheptoses do not give a positive test after double extraction when used in the same concentrations as pyruvate. The method of Burton and San Pietro (7) was used to develop chromatograms in the identification of pyridine nucleotides.

Enzyme Assays and Measurement of Oxidation

Glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase were assayed by standard procedures (35, 31, 38) using phosphopyridine nucleotide reduction as a measure of the reaction rate. The reactions were carried out in a Beckman Spectrophotometer (Model DU) at 340 mμ.

Measurements of the Entner-Doudoroff (17) pathway of 6-PG oxidation were carried out according to the detailed directions published by Kovachevich and Wood (36, 37).

Aerobic oxidation experiments were done in conventional Warburg vessels using KOH papers, air as the gas phase, and a temperature of 27° C., in a total volume of 3.2 ml. Evolution of CO_2 was measured by the two vessel method, with and without KOH.

Blanks have been subtracted from all figures quoted in this paper and substrate was always tipped in from the side arm.

Enzyme Preparations

(*a*) *Sonic Preparations*

These extracts were prepared as described in a previous publication (28) from xylose-grown cells of *Pseudomonas hydrophila* (NRC 492). The cells and the cell-free extracts were always obtained fresh before each experiment and were never stored. Freeze-drying or storage at low temperature always affected the oxidative enzyme systems in an unpredictable manner.

(*b*) *Charcoal-treated Sonic Preparations*

These extracts were prepared as described previously (28) and were used in experiments in which it was desirable to employ pyridine nucleotide-free preparations.

(*c*) *Fractional Centrifugation of Sonic or Charcoal-treated Preparations at Higher Speeds*

This was done in a Spinco preparative centrifuge under refrigerated conditions for periods of one hour. Supernatants were used of preparations centrifuged at: (A) 15,000 r.p.m. (20,000 × *g*), (B) 28,000 r.p.m. (50,000 × *g*), and (C) 40,000 r.p.m. (105,000 × *g*).

(*d*) *Ammonium Sulphate Fractions*

The sonic preparation was fractionated by addition of cold, saturated ammonium sulphate at 0° C. and the samples were allowed to stand 30 min. with gentle stirring between the centrifugation of each successive fraction. Fractions representing 0–30, 30–40, and 40–50% saturation were spun at 8000 r.p.m. (5900 × *g*) whereas fractions of 50–70 and 70–95% saturation were spun at 12,500 r.p.m. (14,000 × *g*). For experiment the ammonium sulphate precipitates were dissolved or suspended in the appropriate volume of 0.04 *M* glycylglycine buffer at pH 7.3.

Experimental and Results

Oxidation of D-*Xylose by Intact Cells*

Cells grown on a simple synthetic medium (29) containing D-xylose as the only source of carbon were found to oxidize this sugar rapidly. For each $\mu M.$ xylose added 2.99 $\mu M.$ oxygen were taken up and 2.45 μM. CO_2 were produced (Fig. 1).

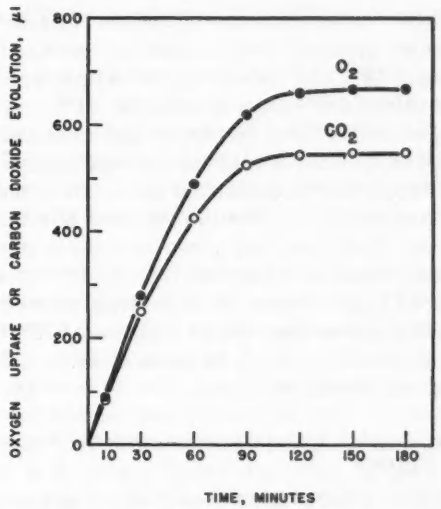

FIG. 1. Oxygen uptake and CO_2 evolution from the oxidation of D-xylose by washed, intact cells. *Components:* 0.022 *M* phosphate buffer, pH 7.0; cell suspension 200 mgm. wet weight; 0.0033 *M* D-xylose.

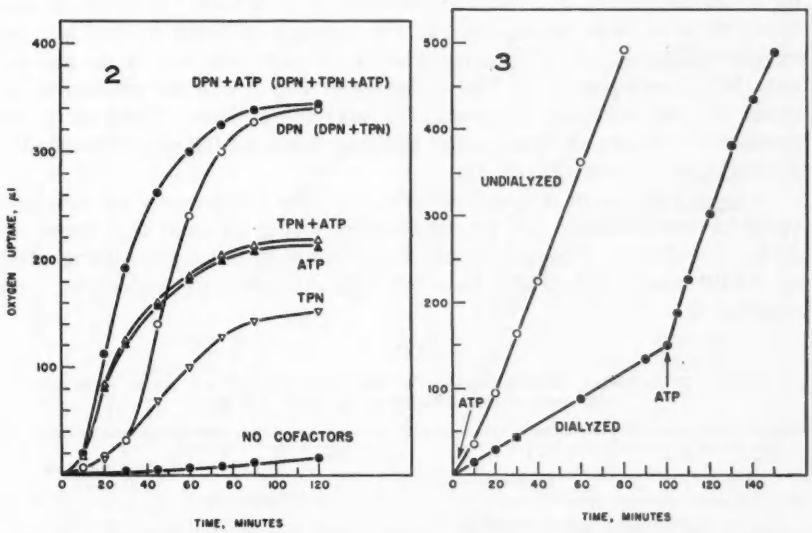

FIG. 2. Oxidation of D-xylose by cell-free, sonic extracts. *Components:* 0.022 *M* phosphate buffer, pH 7.0; 0.8 ml. sonic preparation; 0.003 *M* $MgCl_2$; 0.0008 *M* TPN; 0.0008 *M* DPN; 0.0032 *M* ATP; 0.005 *M* D-xylose.

FIG. 3. Comparison between the oxygen uptakes due to xylose oxidation by undialyzed and dialyzed sonic preparations. (ATP added at the times indicated). *Components:* 0.022 *M* phosphate buffer, pH 7.0; 0.003 *M* $MgCl_2$; 0.00085 *M* DPN; 0.00075 *M* TPN; 0.0032 *M* ATP; 1.0 ml. sonic preparation (treated as shown); 0.022 *M* xylose.

The Effects of Cofactors on the Oxidation of D-*Xylose by Cell-free, Sonic Extracts*

Cell-free extracts oxidized xylose rapidly, provided the appropriate cofactors were added (Fig. 2). Much higher oxygen uptakes were obtained in the presence of added DPN than with added TPN. ATP alone had considerable effect and its addition to the system containing added pyridine nucleotides almost eliminated initial lag periods always encountered with such systems. It appears, therefore, that the extracts themselves contained considerable amounts of the pyridine nucleotides but that additional ATP was also necessary. The latter fact arises from the requirement for ATP in the initial phosphorylation of D-xylulose (26, 27, 48, 51) and because of the presence of some ATP-ase activity in fresh sonic extracts (51). Whenever DPN was the added nucleotide, further addition of TPN did not give rise to any increase in reaction rate. Addition of other cofactors (e.g. FAD, cytochrome *c*) did not alter these results.

The Dependence of Aerobic Xylose Oxidation upon a Phosphate Donor, Divalent Cation, and ThPP

Addition of ATP to sonic preparations dialyzed against distilled water for three hours restored the activity to that of the undialyzed preparation in the presence of pyridine nucleotides and magnesium ions (Fig. 3).

The same experimental system was used to demonstrate the requirement for a divalent metal ion when xylose was the substrate. Magnesium was found to serve most effectively. In the presence of 0.003 *M* $MgCl_2$ a net oxygen uptake of 175 μl. was recorded in 90 min. whereas in its absence only 10 μl. was observed. The oxidation of R-5-P was not affected at all either by the presence or absence of magnesium ions. These data are consistent with the previously established requirement for magnesium in the xylulokinase reaction (29, 26, 27).

In fresh sonic or in dialyzed extracts, no other requirement for cofactors could be demonstrated. If the preparation was lyophilized and stored for 24 hr. at −20° C. its oxygen uptake was diminished but a stimulating effect by ThPP was noted (Table I). CoA did not stimulate under the same conditions.

TABLE I

EFFECT OF THIAMINE PYROPHOSPHATE ON THE OXIDATION OF XYLOSE BY DIALYZED AND UNDIALYZED, FREEZE-DRIED SONIC EXTRACTS

Systems	μl. O_2 uptake in 120 min.
Undialyzed extract (control)	141
Dialyzed extract (control)	42
Dialyzed extract + CoA (0.00028 *M*)	50
Dialyzed extract + ThPP (0.0007 *M*)	148

Common reaction mixture: 0.022 M phosphate buffer, pH 7.0; 0.003 M $MgCl_2$; 0.0009 M DPN; 0.0004 M TPN; 0.0032 M ATP; 0.0007 M ADP; 50 mgm. per ml. freeze-dried enzyme preparation (dialyzed or undialyzed as shown); 0.0033 M xylose.

Effect of High-speed Centrifugation on the Ability of Sonic Extracts to Oxidize Xylose

For routine use, extracts were centrifuged at 7500 r.p.m. (5900 × *g*) as described previously (28). Raising the speed to 28,000 r.p.m. (50,000 × *g*) resulted in a preparation which had much lower turbidity but oxidized xylose almost as well. This preparation was found to be most suitable for the spectrophotometric experiments. Centrifugation at 40,000 r.p.m. (105,000 × *g*) gave a clear extract which was almost totally inactive.

The Efficacy of Charcoal for the Removal of Nucleotides from Extracts and Comparison Between Rates of Xylose and R-5-P Oxidation

It was desirable to obtain enzyme preparations which were free of pyridine nucleotides in order to facilitate study of the pathways involving DPN and TPN. This was achieved by treatment of fresh, cell-free, sonic extracts with charcoal by the method previously described (28). The preparations were found to be sufficiently free of nucleotides that no oxygen uptake occurred unless either DPN or TPN was added (Fig. 4). DPN-catalyzed oxidations always resulted in higher oxygen uptakes than comparable TPN-linked reactions. The amount of nucleotide required to saturate the enzymes fully ranged from 2.2–2.4 μM. DPN or TPN for six different enzyme preparations.

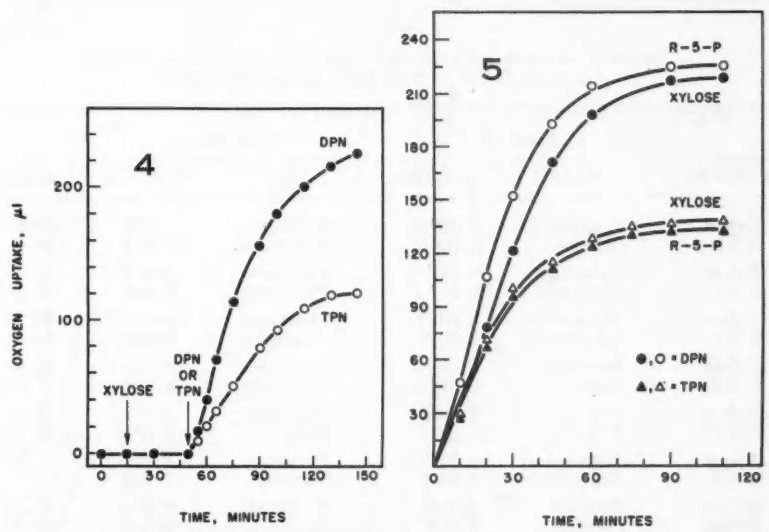

FIG. 4. Xylose oxidation by charcoal-treated extract. *Components:* 0.022 *M* phosphate buffer, pH 7.0; 0.003 *M* $MgCl_2$; 0.0032 *M* ATP; 0.8 ml. charcoal-treated enzyme preparation. *Additions:* at 15 min., 0.0033 *M* xylose; at 50 min., 0.00085 *M* DPN or 0.00075 *M* TPN.

FIG. 5. Comparison between rates of oxidation of xylose and of R-5-P by charcoal-treated extracts in the presence of DPN or TPN. *Components:* 0.005 *M* phosphate buffer, pH 7.4; 0.017 *M* tris, pH 7.4; 0.003 *M* $MgCl_2$; 0.0032 *M* ATP; 0.0009 *M* DPN; 0.00085 *M* TPN; 0.8 ml. charcoal-treated extract; substrates 0.0033 *M*.

Most of the experiments were, therefore, carried out with 2.7–3.0μM. DPN or TPN. The charcoal-treated extracts catalyzed the oxidation of xylose and of R-5-P at identical rates in the presence of either DPN or TPN (Fig. 5). This fact assumes considerable importance when the over-all reaction sequence is considered.

The Effects of Inhibitors on the Oxidation of Xylose and of R-5-P in the DPN-linked Oxidation System

Cyanide, hydrazine sulphate, and iodoacetate were found to be the most effective inhibitors; arsenate and arsenite had little effect and malonate none at all (Table II). This suggests an effect on the triose phosphate oxidation system, and the lack of inhibition by malonate will be discussed later in the light of related work on the tricarboxylic acid cycle. The requirement for phosphate ions to permit demonstration of fluoride inhibition is in accord with established facts concerning the magnesium fluorophosphate complex (54) involved in enolase inhibition. Attention is drawn here to the fact that the effects produced by each inhibitor were the same with either xylose or R-5-P as substrate. The iodoacetate inhibition can be increased by using an extract which has been prepared in the presence of a much smaller quantity of glutathione. Iodoacetate did not inhibit the TPN catalyzed oxidations of the same substrates.

TABLE II

EFFECTS OF INHIBITORS ON THE AEROBIC OXIDATION OF XYLOSE AND OF R-5-P IN CELL-FREE, SONIC EXTRACTS

Substrate (0.0033 *M*)	Inhibitor		Buffer		% Inhibition
	Type	Conc. (*M*)	Type	Conc. (*M*)	
Xylose	Cyanide	0.00065	Phosphate	0.022	100.0
R-5-P	Cyanide	0.00065	Phosphate	0.022	98.0
Xylose	Hydrazine	0.017	Phosphate	0.022	92.4
R-5-P	Hydrazine	0.017	Phosphate	0.022	90.8
Xylose	Iodoacetate	0.0033	Phosphate	0.022	88.5
R-5-P	Iodoacetate	0.0033	Phosphate	0.022	82.3
Xylose	Arsenate	0.001	Phosphate	0.022	22.8
R-5-P	Arsenate	0.001	Phosphate	0.022	15.2
Xylose	Arsenite	0.0033	Phosphate	0.022	18.0
R-5-P	Arsenite	0.0033	Phosphate	0.022	10.2
Xylose	Malonate	0.02	Phosphate	0.022	0.0
R-5-P	Malonate	0.02	Phosphate	0.022	0.0
Xylose	Fluoride	0.048	Phosphate	0.022	49.3
Xylose	Fluoride	0.048	Tris	0.017	0.8
Xylose	Fluoride	0.048	Tris*	0.017	41.8
R-5-P	Fluoride	0.048	Phosphate	0.022	52.3
R-5-P	Fluoride	0.048	Tris	0.017	1.7
R-5-P	Fluoride	0.048	Tris*	0.017	47.2

**In these experiments 0.004 M phosphate was also present.*

Components of reaction mixture common to all vessels: 0.00085 M DPN; 0.0032 M ATP; 0.003 M $MgCl_2$; 0.8 ml. sonic enzyme preparation.

Sedoheptulose-7-phosphate as a Key Intermediate During Aerobic Oxidation of Xylose and R-5-P

Previous work from this laboratory (27, 28) had shown that the anaerobic route of xylose and R-5-P dissimilation in *P. hydrophila* follows the same pathway. If this fact also holds for the aerobic oxidation of these substances, SH-7-P should be an intermediate in both cases. Furthermore, since isomerization and phosphorylation precedes xylose utilization (26) in this organism, it is conceivable that the heptulose ester might appear more quickly when R-5-P is being oxidized.

It was, therefore, decided to study the formation and disappearance of SH-7-P during the oxidation of these substrates. SH-7-P was determined as follows: After aerobic incubation in conventional Warburg vessels for selected time intervals, proteins were precipitated in each sample with TCA and the concentrated supernatants were transferred to filter paper sheets. After development of the chromatograms the heptulose areas were eluted from the papers with water and their concentrations determined by the Dische (16) method. Values so obtained were corrected for the amounts "lost" owing to development of the reference strips.

Results from such experiments with charcoal-treated sonic preparations are shown in Fig. 6. There was a rapid transient formation of sedoheptulose-7-

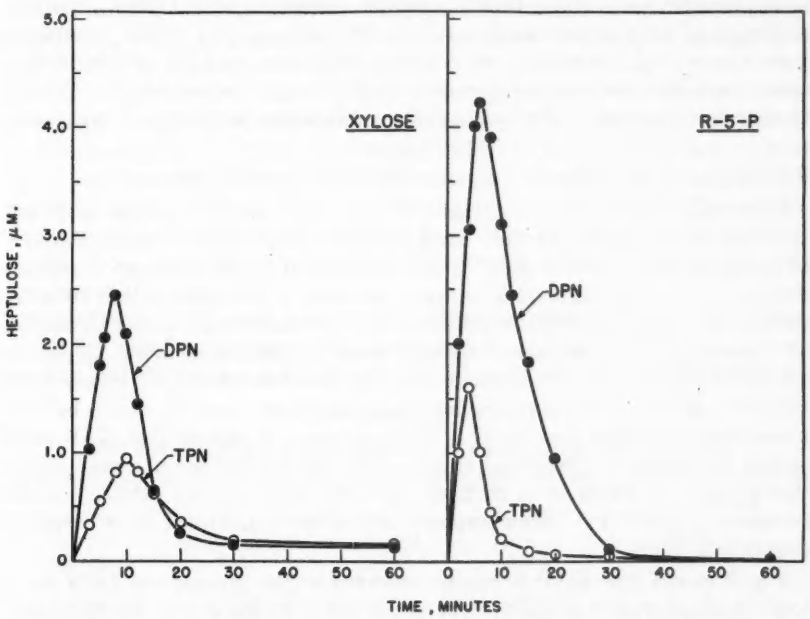

FIG. 6. Heptulose formation and disappearance during aerobic oxidation of 10 μM. xylose and 10 μM. R-5-P in the presence of 0.00085 *M* DPN or TPN. Method of elution and analysis as described in the text. Components of initial oxidation mixture as in FIG. 5.

phosphate (R_f = 0.37 in propanol : acetic acid : water, 60 : 1 : 39, see (27)), which disappeared very quickly. In all cases, heptulose formation reached its maximum faster when R-5-P was the substrate used than with xylose. A comparison of these analytical values with rates of oxygen uptake (see Fig. 5) reveals that the peaks of heptulose formation were always reached well before any appreciable oxygen uptake could be noted. Since heptulose phosphate formation has been demonstrated to lie on the anaerobic pathway (28) and since its formation under aerobic conditions precedes oxygen uptake, it follows that the locus of oxygen uptake lies at a point following the formation and disappearance of heptulose. The TPN-catalyzed oxidations result in much lower heptulose accumulation than do the DPN-linked systems in a manner similar to their oxygen uptakes. The reason for the higher heptulose values when R-5-P was the substrate is not known. Aerobic heptulose accumulation might be explained in several ways. It may be due to a relatively sluggish transaldolase system in these extracts incapable of handling large quantities of heptulose under these conditions. Alternatively, it may also be due to a competition between transaldolase, aldolase, and triose phosphate dehydrogenase (in presence of DPN) for the available GAP which is necessary for transaldolation of SH-7-P. The latter is a good possibility since these extracts contain a very active aldolase (determined by the standard spectrophotometric assay method containing HDP, arsenate, cysteine, and DPN). Since a minimum of six enzymes are involved in the formation of hexose phosphate from xylose and since their relative concentrations, specific activities, and equilibrium constants are not known accurately for these extracts it is unwise to speculate further on the kinetics of sedoheptulose formation at this time.

The Oxidation of Trioses and Triose Phosphates by Sonic Extracts

Since the rates of oxygen uptake were the same whether xylose or R-5-P was used as substrate and since early transient formation and disappearance of heptulose was shown to occur in the oxidation of either substrate it appears evident that the initial aerobic pathway involved is the same as that recently worked out under anaerobic conditions (28). Anaerobically, a net formation of triose- and hexose-monophosphate was demonstrated with xylose as substrate (28). Since the present reaction mixtures contained excess ATP and since the enzymes necessary for triose phosphate formation from hexose-monophosphate were also found in these extracts, it follows that GAP itself should be formed in the initial stages. The oxygen uptake observed might then be due, in whole or in part, to the GAP being rapidly oxidized in the presence of DPN by triosephosphate dehydrogenase linked to a terminal respiratory chain.

Fig. 7 shows that GAP is readily oxidized in the presence of DPN by a fresh charcoal-treated extract. The initial reaction rate is considerably faster than with xylose. This is to be expected if GAP is itself the substrate of the terminal oxidation system; in fact, it is noteworthy that 10 μM. xylose give rise to the same final oxygen consumption as do 10 μM. GAP. The oxidation

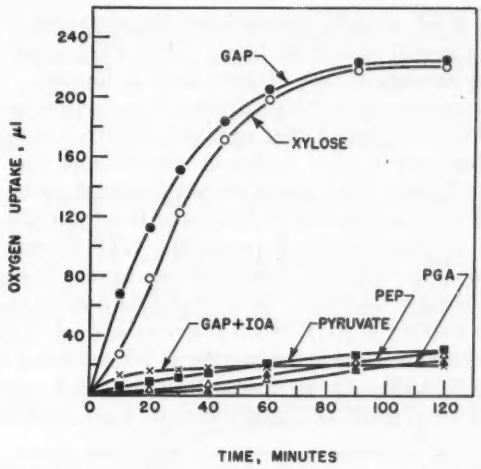

FIG. 7. Comparison between the rates of oxidation of xylose, GAP, and other trioses. *Components:* 0.017 *M* tris, pH 7.4; 0.004 *M* phosphate buffer, pH 7.4; 0.003 *M* $MgCl_2$; 0.0032 *M* ATP; 0.0009 *M* DPN; 0.8 ml. charcoal-treated extract; concentration of all substrates 10 µM.; IOA (where used) 0.005 *M*.

of GAP is very sensitive to IOA. No significant oxygen uptake was obtained with PGA, PEP, or pyruvate. When the system was fortified further with TPN, ATP, ADP, CoA, and α-lipoic acid there was still no significant oxygen uptake. Furthermore none could be demonstrated with acetate, acetyl phosphate, oxalacetate, and combinations of pyruvate and acetyl phosphate with oxalacetate in systems which were fortified as mentioned above. Under these particular experimental conditions oxygen uptake due to reactions of the tricarboxylic acid cycle and possible other sources is eliminated quantitatively since our results show that 1 mole O_2 is consumed per mole of either xylose, R-5-P, or GAP. Oxidation at the triosephosphate level fully accounts for the results obtained.

Oxidation of GAP was followed up by a study of possible PGA accumulation under the same experimental conditions as used in Fig. 7. In Table III are summarized the oxygen uptakes and the amounts of GAP and PGA formed.

TABLE III

GAP OXIDATION AND PGA FORMATION BY CHARCOAL-TREATED EXTRACTS

Time (min.)	Net oxygen uptake (µl.)	GAP residual (µM.)	PGA formed (µM.)	PGA formed in presence of 0.08 *M* NaF (µM.)
0	0	10.0	0.0	0.0
20	112	5.1	1.8	4.1
40	175	2.35	1.4	6.8
60	205	0.92	0.8	8.1
120	224	0.1	0.3	8.8

Relatively little PGA actually accumulated but the small amount which did accumulate disappeared again with time. Thus, PGA appears to be removed by an anaerobic process almost as soon as it is formed. Its accumulation can be demonstrated however by using fluoride to inhibit enolase.

The above results suggested that pyruvate might accumulate under these conditions. The results of a series of experiments set up to test this possibility (Fig. 8) show that pyruvate accumulated quite early during the oxidation of xylose (maximum at 12 min.) and R-5-P (10 min.) and also when PGA was used as substrate (four minutes). ATP is required for xylulose phosphorylation (Fig. 8A) and the ADP so formed serves as phosphate acceptor in the enolase reaction. When R-5-P is used as substrate the ATP serves merely as a source of ADP which is formed in fresh extracts by ATP-ase. The identical curve was obtained when the R-5-P oxidizing system contained ADP in place of ATP. In all cases, NaF inhibited pyruvate formation strongly. PGA was itself not oxidized but was transformed quickly to

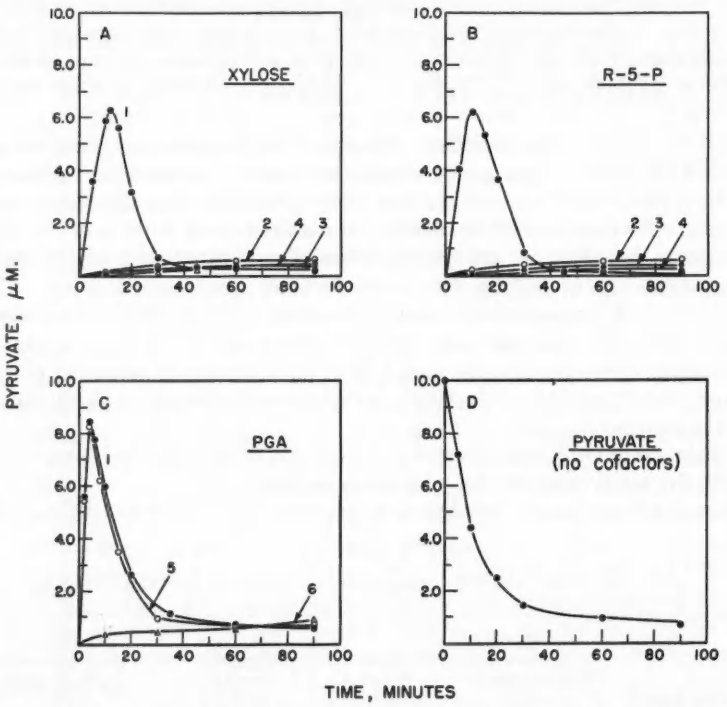

FIG. 8. Formation and disappearance of pyruvate during the aerobic metabolism of 10 μM. of xylose, R-5-P, or PGA (A, B, C, respectively) by charcoal-treated extracts in presence of DPN. The rate of pyruvate disappearance without any added cofactors (D). 1 = substrate (S) + ATP + DPN; 2 = S + ATP; 3 = S + DPN; 4 = S + ATP + DPN + NaF; 5 = S + ADP; 6 = S + ADP + NaF. *Concentrations:* 0.017 *M* tris, pH 7.4; 0.005 *M* phosphate buffer, pH 7.4; 0.004 *M* $MgCl_2$; 0.003 *M* ATP (or ADP where indicated); 0.0009 *M* DPN; 0.07 *M* NaF; 0.8 ml. charcoal-treated extract.

pyruvate, which then disappeared also by a pathway which did not lead to oxygen uptake. The rate of pyruvate disappearance itself is shown in Fig. 8D. Addition of such cofactors as DPN, TPN, ATP, ADP, UTP, α-lipoic acid, and CoA had little, if any, effect on the rate of pyruvate disappearance. The mechanism of this reaction remains to be worked out.

Pyridine Nucleotide Specificities of the Dehydrogenases Implicated in DPN- and TPN-linked Oxidations

Results so far presented have shown that both xylose and R-5-P are oxidized by DPN- and by TPN-linked pathways. To investigate these further it was desirable to establish the pyridine nucleotide specificities for the

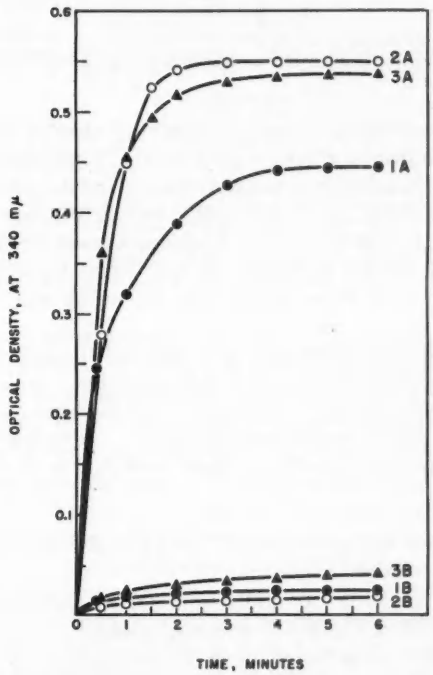

FIG. 9. Pyridine nucleotide specificities of glyceraldehyde phosphate (1A, 1B), glucose-6-phosphate (2A, 2B), and 6-phosphogluconate (3A, 3B) dehydrogenases in soluble extracts of *P. hydrophila*.

Assay systems (3 ml. final volume):

(1A) 0.02 *M* sodium pyrophosphate (pH 8.5); 0.013 *M* cysteine. HCl; 0.013 *M* sodium arsenate; 0.002 *M* GAP; 0.2 ml. enzyme preparation *c*–B (see "Methods"); incubation time seven minutes; final addition, 0.000083 *M* DPN.

(1B) As in 1A, except that final addition was 0.000083 *M* TPN.

(2A) 0.01 *M* glycylglycine (pH 7.4); 0.003 *M* $MgCl_2$; 0.004 *M* G-6-P; 0.1 ml. enzyme preparation *c*–B (no prior incubation); final addition, 0.0001 *M* TPN.

(2B) As in 2A, except that final addition was 0.0001 *M* DPN.

(3A) 0.01 *M* glycylglycine (pH 7.4); 0.003 *M* $MgCl_2$; 0.004 *M* 6-PG; 0.4 ml. enzyme preparation *c*–B (no prior incubation); final addition, 0.0001 *M* TPN.

(3B) As in 3A, except that final addition was 0.0001 *M* DPN.

TABLE IV

PYRIDINE NUCLEOTIDE SPECIFICITIES OF GAP, G-6-P, AND 6-PG DEHYDROGENASES IN AN AMMONIUM SULPHATE FRACTION (0.5–0.7 SATURATION) FROM CHARCOAL-TREATED, CELL-FREE EXTRACTS OF *P. hydrophila*

Substrate	Pyridine nucleotide used	Net optical density after 5 min. reaction time
GAP	DPN	0.435
GAP	TPN	0.008
G-6-P	DPN	0.000
G-6-P	TPN	0.532
6-PG	DPN	0.032
6-PG	TPN	0.540

Assay conditions for each system exactly as described in Fig. 9. 0.3 ml. enzyme preparation used (3 mgm. protein/ml.).

dehydrogenases responsible for the oxidations of GAP, G-6-P, and 6-PG in *P. hydrophila*. The results of spectrophotometric determinations are given in Fig. 9. They show that the glyceraldehyde phosphate dehydrogenase is specific for DPN and that both G-6-P and 6-PG dehydrogenases react only with TPN. Thus, these *P. hydrophila* dehydrogenases resemble similar enzymes of animal tissues and certain bacteria but are unlike those from *Leuconostoc mesenteroides* (14, 13) or from plants (19, 45) which will work with either DPN or TPN.

Ammonium sulphate fractionation of a charcoal-treated cell-free extract of *P. hydrophila* yielded a fraction (0.5–0.7 saturation) which contained the highest dehydrogenase activity. When this fraction was used in place of the fresh extract very similar results were obtained. The specificities obtained (Table IV) corresponded closely to those observed with the fresh extract (Fig. 9).

The Differential Effect of Iodoacetate on Xylose and R-5-P Oxidation as Studied Spectrophotometrically

As may be seen from Fig. 10, addition of DPN, arsenate, and cysteine to either the xylose-ATP or the R-5-P systems resulted in immediate and rapid DPN reduction, which was found to be quite sensitive to IOA. TPN was also reduced most effectively but only after a short period of incubation with the enzyme preparation. The lag period shown in the system in which measurements were begun immediately is interpreted as representing the time required for pentose cycle substrate (i.e. G-6-P) formation through the isomerase–epimerase–transketolase–transaldolase series of reactions. A strictly analogous experiment with DPN could not be carried out since some incubation of enzyme with cysteine and arsenate (and IOA, where used) is necessary for maximum glyceraldehyde phosphate dehydrogenase activity (38). The insensitivity of the TPN-linked system to IOA is, however, readily seen in both experiments.

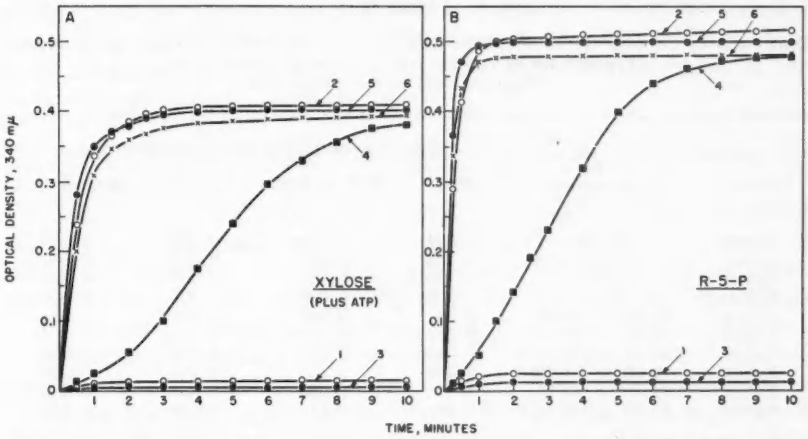

FIG. 10. Oxidation of xylose and of R-5-P by *P. hydrophila* extracts as measured spectrophotometrically by pyridine nucleotide reduction. For experiments A and B: 1 = DPN; 2 = DPN + arsenate; 3 = DPN + arsenate + IOA; 4 = TPN (15 sec. incubation after enzyme addition); 5 = TPN (15 min. incubation after enzyme addition); 6 = TPN + IOA. *Concentration of components.*—(*a*) TPN system: 0.2 ml. enzyme preparation *c*–B; 0.02 *M* glycylglycine buffer (pH 7.35); 0.012 *M* xylose or R-5-P (as indicated); 0.000083 *M* DPN or TPN (as indicated); 0.0066 *M* $MgCl_2$; 0.008 *M* IOA (only where indicated); 0.013 *M* ATP (experiment A only); (*b*) DPN system: in addition to (*a*), 0.013 *M* cysteine and 0.013 *M* arsenate.

From these data it is suggested that when DPN is used as cofactor in the oxidation of both xylose and of R-5-P, oxidation proceeds via the DPN-specific glyceraldehyde phosphate dehydrogenase to the terminal respiratory carrier chain. When TPN is used oxidation most probably takes place via G-6-P dehydrogenase by a pathway which is quite insensitive to IOA.

Very similar data were obtained with ammonium sulphate fractions of the enzyme preparation (Table V). The DPN-linked system was strongly inhibited by IOA whereas the TPN-catalyzed reaction was completely unaffected by the same concentration of the inhibitor. Table V further indicates that the complete oxidation system working either through DPN or TPN is found in the soluble fraction (50–70% saturation) when R-5-P is used as the substrate. When xylose is used, however, some activity resides also in the fraction precipitated between 40–50% saturation. The best results were obtained when the two fractions were combined resulting in values which were quite comparable to those obtained with R-5-P as substrate. This suggests that one or more enzymes catalyzing reactions that lie between xylose and R-5-P are of a less soluble nature (i.e. they precipitate between 35 and 50% saturation (25)).

Formation of Pentose and Heptulose Esters from Hexose Phosphates

Incubation of 6-PG, G-6-P, and HDP plus IOA in the presence of charcoal-treated extracts, tris buffer, TPN, and $MgCl_2$ resulted in the formation of

TABLE V

OXIDATION OF XYLOSE AND OF R-5-P BY AMMONIUM SULPHATE FRACTIONS OF *P. hydrophila* EXTRACTS AS MEASURED SPECTROPHOTOMETRICALLY BY PYRIDINE NUCLEOTIDE REDUCTION

Substrate	Fractions % saturation	Optical density after 6 min. reaction time			
		TPN	TPN + IOA	DPN	DPN + IOA
Xylose	30–40	0.000	0.004	0.000	0.000
Xylose	40–50	0.084	0.079	0.098	0.008
Xylose	50–70	0.171	0.184	0.202	0.021
Xylose	70–95	0.000	0.000	0.000	0.000
Xylose	30–40 + 40–50	0.082	0.088	0.082	0.004
Xylose	30–40 + 50–70	0.201	0.198	0.201	0.015
Xylose	30–40 + 70–95	0.004	0.000	0.000	0.000
Xylose	40–50 + 50–70	0.450	0.451	0.461	0.095
Xylose	40–50 + 70–95	0.074	0.080	0.086	0.008
Xylose	50–70 + 70–95	0.169	0.174	0.178	0.012
R-5-P	30–40	0.000	0.000	0.000	0.000
R-5-P	40–50	0.000	0.008	0.014	0.007
R-5-P	50–70	0.424	0.449	0.427	0.034
R-5-P	70–95	0.012	0.004	0.000	0.007
R-5-P	30–40 + 40–50	0.000	0.000	0.012	0.000
R-5-P	30–40 + 50–70	0.430	0.451	0.420	0.028
R-5-P	30–40 + 70–95	0.008	0.009	0.004	0.007
R-5-P	40–50 + 50–70	0.445	0.439	0.435	0.042
R-5-P	40–50 + 70–95	0.010	0.000	0.004	0.000
R-5-P	50–70 + 70–95	0.438	0.440	0.429	0.030

Components as indicated in legend to Fig. 10 with ammonium sulphate fractions in place of fresh enzyme preparations.

TABLE VI

TRANSFORMATION OF HEXOSE PHOSPHATES TO PENTOSE AND HEPTULOSE ESTERS IN EXTRACTS OF *P. hydrophila*

Substrate (10 μM.)	Time (min.)	Pentose formed (net, μM.)	Heptulose formed (net, μM.)
6-PG	0	0.0	0.0
	10	2.9	0.9
	30	5.9	3.4
	60	3.2	2.0
	120	2.1	1.2
G-6-P	0	0.0	0.0
	10	2.7	0.8
	30	5.4	3.6
	60	2.8	1.9
	120	1.9	0.9
HDP + IOA	0	0.0	0.0
	10	2.4	0.7
	30	4.6	2.7
	60	3.1	1.9
	120	1.2	1.3

pentose and heptulose esters as shown in Table VI. Since the complete transformations of xylose and of R-5-P to hexosemonophosphate have been found previously in this organism (28), the above demonstration of the remaining reactions completes the cycle.

The Entner-Doudoroff Pathway as a Possible Factor in the Interpretation of the Over-all Oxidative Pattern

It was shown in Fig. 8 that during the early phases of xylose and R-5-P oxidation, pyruvate was formed but disappeared rapidly. The work of Entner and Doudoroff (17) and of Kovachevich and Wood (36, 37) has shown that 6-PG can give rise to equimolar quantities of GAP and pyruvate. Since GAP oxidation was one of the key phases of the present study it was important to investigate the possibility of its formation via 6-phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate aldolase. Assay conditions used were exactly as described by the latter authors (36, 37). In most experiments pyruvate values obtained with the charcoal-treated enzyme preparation were approximately 0.3–0.6 μM. from 10 μM. of 6-PG. In only two cases were values as high as 1.1 μM. encountered. Dialyzed extracts fortified with GSH and $FeSO_4$ yielded approximately 0.12–0.28 μM. pyruvate. The usual concentrations of hydrazine and arsenite were employed. Only trace amounts of GAP could be demonstrated. Thus it would appear that this pathway is of little significance in the evaluation of the over-all oxidative patterns studied in *P. hydrophila*.

Possible Pyridine Nucleotide Interactions

Kornberg (34) first purified an enzyme from yeast which catalyzes the synthesis of TPN by a direct phosphorylation of DPN by ATP. If this enzyme were present in our preparations it would make interpretation of the DPN- and TPN-linked pathways far more difficult. The method chosen to test this possibility was incubation with the charcoal-treated sonic preparation of DPN, TPN, DPN + ATP, and TPN + ADP using the exact pH and cofactor conditions published by Kornberg (34). Following incubation for 0, 5, 20, and 60 min. periods, TCA was added to each sample and the supernatants following a short period of centrifugation were spotted on filter papers so that each spot contained 100 μgm. of pyridine nucleotide. Only the nucleotide originally incubated was found on the chromatograms. An enzymatic test using glucose-6-phosphate dehydrogenase to test an aliquot of the incubation mixture of DPN + ATP also proved negative. Thus, there was no evidence of any phosphorylation of DPN to give TPN.

Another possibility of pyridine nucleotide interactions was considered to be the transhydrogenase first found by Colowick *et al.* (10). This enzyme catalyzes the reaction: TPNH + DPN $\rightarrow$ TPN + DPNH. Thus, in the presence of such an enzyme data concerning the specificity of a nucleotide in a given reaction may become questionable. A charcoal-treated, fresh enzyme preparation, spun at 10,000 r.p.m. for 40 min., was used for the experiment.

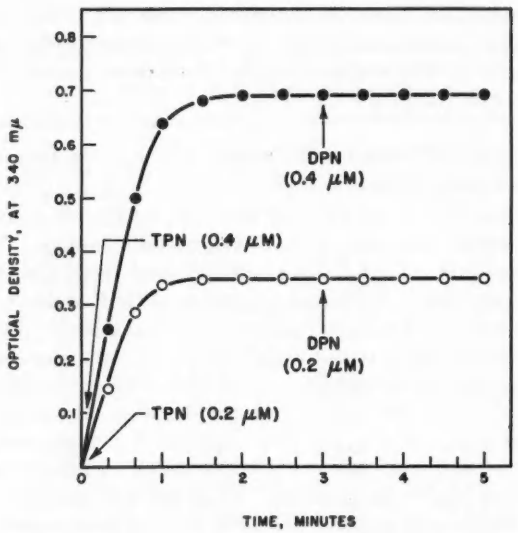

FIG. 11. Assay of *P. hydrophila* extracts for pyridine nucleotide transhydrogenase activity using G-6-P dehydrogenase as the TPNH-generating system. *Components:* quantities of nucleotides as indicated, 0.002 *M* G-6-P, 0.003 *M* $MgCl_2$, 0.03 *M* glycylglycine buffer (pH 7.1), 0.3 ml. charcoal-treated enzyme preparation (centrifuged at 10,000 r.p.m.).

The TPN-specific glucose-6-phosphate dehydrogenase system was used for the generation of reduced TPN in the presence of excess G-6-P. It was already shown that DPN is not acted upon at all (Fig. 9) by this enzyme in *P. hydrophila*. As Fig. 11 shows, TPN is rapidly reduced, the reduction being complete when an excess of substrate is used. After complete reduction of TPN by excess G-6-P, addition of DPN did not alter the optical density values. If the transhydrogenase had been present this would have resulted in an additional increment in absorption at 340 mμ corresponding to the complete reduction of the added DPN.

It is, therefore, possible to interpret the results obtained in this investigation without further consideration of these enzymes.

Discussion

From the literature now available concerning the nucleotide specificities of pentose phosphate oxidations it appears that not all organisms follow the same pattern. Some differences in DPN and TPN specificities have already been discussed. In addition to these, several recent papers should be mentioned. Claridge and Werkman (8) have found R-5-P to be capable of reducing DPN but not TPN in *P. aeruginosa* in contrast to similar work of Glock (20), who obtained the opposite results with liver preparations. Mortenson and Wilson

(42) demonstrated the inability of R-5-P to reduce DPN in *A. vinelandii* extracts and Cochrane *et al.* (9) found TPN superior as a hydrogen acceptor. Differences in the specificities of the G-6-P and 6-PG dehydrogenases have also been recorded (23, 55) in addition to those already referred to. Further purification and amino acid analysis of these enzymes will be necessary before a more thorough understanding of the exact nature of these specificities is possible. Subtle structural differences in the enzymes obtained from different biological sources probably exist.

The data recorded here for *P. hydrophila* differ in many respects from those published for *A. vinelandii* (42) extracts. In the latter case phosphate was found to inhibit and fluoride to stimulate R-5-P oxidation. Although not mentioned in the experimental portion of the present paper, oxygen uptake values with phosphate buffer as compared to those with tris showed few significant differences whereas fluoride was always found to inhibit provided some phosphate was also present. Also, in the case of *P. hydrophila* extracts, succinate added to R-5-P did not stimulate its oxidation, nor did malonate inhibit (see Table II). This may be taken as additional evidence for the non-functioning of the tricarboxylic acid cycle under our conditions although the individual enzymes of the cycle appear to be present.

The apparent absence of the Entner-Doudoroff pathway, DPN-kinase, and pyridine nucleotide transhydrogenase from our preparations is also noteworthy particularly since the former pathway has been found operative in many *Pseudomonas* species.

Vandemark and Wood (52) have just published their results of glucose metabolism studies with *Microbacterium lacticum*. In their system (in the presence of DPN) R-5-P is oxidized with the formation of an equivalent amount of pyruvate which does not disappear again as it does in our case. These authors also note that pyruvate is not formed via the Entner-Doudoroff pathway in this organism and that the TPN-linked oxidation of R-5-P results in much lower oxygen uptakes than with DPN. These latter points agree with our data for *P. hydrophila*.

Since the DPN- and the TPN-linked oxidation systems for xylose and R-5-P exist side by side in *P. hydrophila*, even though the mechanisms of the reactions of which they are a part differ, it is suggested that in the intact cell the two pathways compete with one another for the available substrate materials. A study of such a competition would become feasible if Newburgh and Cheldelin's recent suggestion (44) of a possible intimate association of the pentose cycle enzymes in the form of a small organelle should find experimental verification.

Acknowledgment

We wish to express our appreciation to Mrs. A. Harvey for her most valuable technical assistance.

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CHANGES IN THE YEAST POPULATION ON QUEBEC APPLES DURING RIPENING[1]

By Audrey J. Williams[2], R. H. Wallace, and D. S. Clark

Abstract

The numbers and species of yeasts occurring on Quebec apples were determined at two-week intervals during the ripening season. The numbers of yeasts decreased during August but increased in October. Ten species were isolated of which *Candida malicola* and *Rhodotorula glutinis* var. *rubescens* were the most predominant. There was not any evidence to show that rainfall was correlated with the numbers of yeasts on apples.

Very few studies have been reported about changes in the microbial population of fruits; indeed, publications of this nature concerning apples have been particularly scarce. The only previous report about the changes of microorganisms occurring on apples during the growing season seems to have been made by Marshall and Walkley (4). They found that the numbers of yeasts increased during growth and ripening of English apples in July, August, and September, but that they decreased in October. They did not describe changes in the types of yeasts.

Clark *et al.* (2) have described the occurrence of predominant yeasts of apples grown in the Province of Quebec. In a study of 50 isolated yeasts, they found that all were members of the family Cryptococcaceae, and that the most abundant species was one which had been named *Candida malicola* (1). The apples used for the work were ripe and had been collected from the orchards during October. The purpose of the work reported here was to determine possible changes in the numbers of yeasts and in the occurrences of predominant species on Quebec apples during the ripening season.

Experimental Methods

Apples were gathered from orchards in three districts of Quebec, namely, Rougemont, Franklin Centre, and Macdonald College, at two-week intervals, from August to October, 1954. The Rougemont apples were varieties of McIntosh and Fameuse; those from Franklin Centre were of mixed varieties; and those from Macdonald College were McIntosh. Five samples were gathered aseptically in sterile glass dishes each time from each of the three orchards. Each sample consisted of at least five apples. The laboratory procedure for plating and isolating the yeasts was essentially the same as that used by Clark *et al.* (2), except that the plating medium was one devised by Wickerham (5). The numbers of yeasts were recorded and representative

[1]*Manuscript received July 23, 1956.*
Contribution from the Department of Bacteriology, Macdonald College, Macdonald College P.O., Que. Macdonald College Journal Series No. 390.
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colonies of the predominating yeasts were selected for classification studies. Over 200 yeasts were isolated, purified, and maintained on Wickerham's malt extract yeast extract medium (5) containing sufficient agar to make it semisolid.

The following morphological and physiological characteristics of the isolated yeasts were studied: shape and size of cells; type of vegetative reproduction; ascospore formation; number and shape of blastospores and ascospores; macromorphological characteristics; pellicle formation; carbon assimilation; fermentation; production of starchlike compounds; splitting of arbutin; reaction of litmus milk; production of acid; nitrogen assimilation; and pigmentation. All tests were done in duplicate. The incubation temperature was 25° C. The yeasts were identified in accordance with the classification scheme of Lodder and Kreger-van Rij (3).

Results

Numbers

The average numbers of yeasts from apples of the respective orchards are shown in Table I.

TABLE I

AVERAGE NUMBERS OF YEASTS FROM APPLES OF THREE ORCHARDS IN QUEBEC (PER GRAM OF APPLE)

Date, 1954	Rougemont	Franklin Centre	Macdonald College
Aug. 2	19,900	17,800	17,500
Aug. 16	13,300	9,700	5,900
Aug. 31	3,100	5,200	4,200
Sept. 13	2,900	3,700	2,500
Sept. 27	3,400	1,700	3,100
Oct. 11	12,600	—	8,000

It can be seen that the numbers of yeasts from apples of the three orchards were about the same at the beginning of the investigation. There was, however, a sharp decrease during August, which appeared sooner with those of the Macdonald College apples. This decrease was to a level that remained fairly constant through September for the Rougemont and Macdonald College apples. Particularly low numbers were present on the Franklin Centre apples, however, at the end of September. In October, there was a noticeable increase on apples from two of the orchards; by this time the fruit at Franklin Centre had been harvested and samples could not be obtained. These results are the opposite of those obtained by Marshall and Walkley (4).

Types

Between August 16 and October 11, 222 predominant yeasts were isolated from 75 samples. On classification it was found that they could be grouped into six genera and 10 species. Their names and the numbers of each species isolated according to date are shown in Table II.

TABLE II

NUMBERS OF ISOLATED SPECIES OF YEASTS FROM QUEBEC APPLES AT VARIOUS SAMPLING TIMES

Species	Aug. 16	Aug. 31	Sept. 13	Sept. 27	Oct. 11	Total
Candida malicola	16	14	35	29	17	111
Rhodotorula glutinis var. *rubescens*	5	4	17	5	5	36
Cryptococcus laurentii	0	4	7	5	3	19
Torulopsis famata	2	1	4	1	7	15
Rhodotorula mucilaginosa	4	3	3	2	2	14
Torulopsis candida	0	2	4	1	5	12
Candida krusei	0	0	3	2	0	5
Cryptococcus albidus	0	2	3	1	0	6
Kloeckera apiculata	0	0	0	1	2	3
Saccharomyces bisporus	0	0	1	0	0	1

It is to be noted that 111, or 50%, of the isolated yeasts were *Candida malicola*, which was most abundant during September, and which had been found previously to be predominant on Quebec apples (1). *Rhodotorula glutinis* var. *rubescens* was next in abundance, but only comprising about 16% of the total cultures; it seemed to be especially predominant in mid-September. *Cryptococcus laurentii*, *Torulopsis famata*, *Rhodotorula mucilaginosa*, *Torulopsis candida*, and *Candida krusei*, as a group, were next in order of frequency with generally similar occurrences throughout the sampling time. Lastly, *Cryptococcus albidus*, *Kloeckera apiculata*, and *Saccharomyces bisporus* together composed only about 4½% of the isolated yeasts.

Discussion

Clark *et al.* (2) and Marshall and Walkley (4) have suggested that noticeable decreases in the numbers of yeasts on apples may be attributed to rainfall. During this investigation metereological data were obtained for the three districts in which the three orchards were located. The rainfall in each of the three areas during July was less than two inches; during August, 2–3 in.; during September, 2 in. at Franklin Centre, but 5 and 6 in. at

Macdonald College and Rougemont, respectively. The slight increase in rainfall during August did not seem to be sufficient for correlation with the large reduction of yeasts on the apples. Furthermore, the relatively excessive rainfall at Macdonald College and Rougemont during September did not bring about a noticeable change in the numbers of yeasts on the apples of those districts as compared with Franklin Centre. There is insufficient evidence from the results of this work, therefore, to support the suggestions made by previous workers in respect of rainfall and numbers of yeasts on apples.

Six of the eight species of yeasts formerly isolated by Clark *et al.* (2) from Quebec apples were also isolated during this investigation. There were, in addition, five other species that were not found in the previous work. It is concluded that most of the yeasts named in Table II represent those indigenous to Quebec apples throughout the ripening season. It is interesting to note, in support of previous findings (2), that most of the yeasts isolated were members of the family Cryptococcaceae. It appears also that fermenting-type yeasts were not predominant on the fruit; indeed, only one species of the genus *Saccharomyces* was obtained throughout the work, and this was isolated at only one of five sampling times. Evidence that fermenting-type yeasts were in fact present at other sampling times, but obviously in very low numbers, was obtained only by placing 10 ml. suspensions of comminuted apples into a flask of medium appropriately stoppered to encourage fermentation.

Acknowledgments

The authors express their thanks to the Quebec Agricultural Research Council for its financial support of the project; and to Mr. F. Dufour, Franklin Centre, Que., and Mr. G. A. Baillargeon, Rougemont, Que., for access to their orchards.

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HYPHAL ANASTOMOSIS AND CYTOLOGICAL ASPECTS OF STREPTOMYCES SCABIES[1]

By K. F. Gregory

Abstract

Two strains of *Streptomyces scabies* and a *Streptomyces* sp., strain T12, have been studied for hyphal anastomosis and its cytological consequences. The presence of multiple short hyphal bridges between neighboring hyphae and the absence of demonstrable cell walls or membranes at the points of contact are presented as evidence for anastomosis. The formation of "initial cells", as a result of hyphal fusion, was not observed. Two types of swollen bodies, deeply stained by the acid-Giemsa method, were noted. One type, identified as the residue of the parent spore, passed through a stage during which it did not stain by this method but subsequently became highly chromatinic. The second type was always terminal and developed concurrently with or subsequent to "secondary mycelium". Aerial hyphae were observed to arise directly from "primary mycelium" in *S. scabies*. Hyphae with cytological characteristics intermediate between primary and secondary types were observed in *Streptomyces* sp. T12.

Introduction

Genetic recombination in *Streptomyces coelicolor* Reiner-Müller (15) and the production of heterokaryons in several other species of *Streptomyces* (1, 16) have been reported without comment as to the mechanism by which nuclei from two parental strains may become associated within a single hypha. Similar phenomena among the filamentous fungi have been ascribed to hyphal anastomosis (13). Cytological demonstration of anastomosis in the streptomycetes, however, is equivocal. Carvajal (2) reported observing hyphal fusions during electron microscope studies of *Streptomyces griseus* but did not publish supporting photographs. Klieneberger-Nobel (8) assumed that fusion occurred within "nests" of hyphae or agglomerations of hyphae described as skeins, network, and scrolls. She observed the development of new cellular elements at the points where two filaments were in close contact. These new bodies were designated "initial cells" since they elongated and divided to form a "secondary mycelium". This secondary mycelium possessed deeply staining chromatinic cylinders, easily stainable transverse septa, and fewer side branches than the "primary mycelium". The secondary mycelium ultimately gave rise to conidia. Erikson (4) challenged these interpretations on the grounds that the "nests" described resulted from branches of hyphae sticking together when the slides were fixed and dried. Furthermore, she stated that a general absence of anastomosis is one of the characteristic features of the streptomycetes, wherein they differ most markedly from fungi. Pénau *et al.* (12) could find no clear cases of anastomosis in *S. griseus*.

Some workers have observed swellings in the hyphae of streptomycetes, not originating from "nests" of tangled filaments, which they considered to be initial cells (3, 9, 17). Some of these structures were found to be intercalary

[1]*Manuscript received July 4, 1956.*
Contribution from the Department of Bacteriology, Ontario Agricultural College, Guelph, Ontario. The investigation was part of the program of the Ontario Potato Scab Research Committee.

while others were attached to the hyphae by short stalks. Dickenson and MacDonald (3) published electron micrographs which were intended to show hyphal anastomosis and the formation of initial cells via anastomosis. These illustrations are not entirely convincing since the hyphal strands are merely shown to lie in contact with one another, and proof of actual fusion is lacking. The work reported here was undertaken to explore further the presence or absence of hyphal anastomosis in streptomycetes and its proposed role in initial cell formation.

Materials and Methods

Streptomyces scabies (Thaxter) Waksman & Henrici, O.A.C. strains A26 and A30, originally isolated from potato scab lesions and purified by single spore isolations with the de Fonbrune micromanipulator, were examined. A *Streptomyces* sp., strain T12, in which initial cells have been described (9), was kindly supplied by Dr. H. B. Newcombe, and was also studied.

The inocula used were prepared by suspending the sporulated surface growth of cultures, five to seven days old, in sterile 1 : 5000 Tween 20[2] and filtering these conidial suspensions through sterile No. 1 Whatman filter papers to remove mycelial fragments. The cultures were grown on the surface of sterile cover slips either by the inverted agar-block technique of Klieneberger-Nobel (8) or in liquid medium spread over the surface of the cover slip. All cultures were incubated at 28° C. over distilled water in a sealed chamber. The high humidity in this chamber prevented evaporation of the media. In some cases, the inverted agar-block cultures were removed from the humidity chamber after 48 hr. incubation so that slow drying would favor sporulation. A yeast-beef broth[3] and two asparagine–glucose media (6, 9) were employed.

Slides were removed from the incubator at hourly intervals for the first eight hours, at four hour intervals for the next 28 hr., and at eight hour intervals subsequently for a total of five days. The growth on each cover slip was allowed partially to dry in air and was fixed for two minutes in the vapor of 2% osmic acid. The agar blocks were then removed from the inverted agar-block cultures and the cover slips immersed in mercuric chloride (saturated) for four minutes.

Three methods of staining were used with all ages of cultures. The Robinow (14) acid-Giemsa method of nuclear staining was followed. The cover slips were immersed in *N* HCl at 60° C. for eight minutes, rinsed with distilled water, and stained with Giemsa stain (20 drops of stock solution per 10 ml. phosphate buffer, pH 7.0) for 20 min. Permanent mounts were made in a mixture of mineral oil and α-bromonaphthalene according to the procedure of Minsavage (10). The tannic acid – crystal violet stain for membranes, as applied by Klieneberger-Nobel (8), was used to reveal

[2]*A wetting agent obtained from the Atlas Powder Co., Wilmington, Delaware, U.S.A.*
[3]*Yeast extract, 3.0 gm.; beef extract, 1.5 gm.; peptone, 6.0 gm.; glucose, 1.0 gm.; distilled water, 1000 ml.; pH 6.5.*

septations. These preparations were also mounted by the Minsavage procedure. One minute in ammonium oxalate – crystal violet (7) provided a clear picture of gross morphology.

Photomicrographs were made with a Leitz Ortholux microscope using a × 90 apochromatic objective, × 12 periplanatic eyepiece, 50 watt low voltage tungsten filament for illumination, Kodak Wratten Filter #58 (green), Leitz extensible mirror reflex camera for plates 9 × 12 cm., and Kodak Ortho-X film or, for low contrast material, Kodalith Ortho-Type 2 film.

Results

Demonstration of Anastomosis

An examination of young (less than 24 hr.) mycelia of the strains of *S. scabies* and, to a lesser extent, the mycelium of *Streptomyces* sp. T12, stained by the simple crystal violet stain, revealed many instances wherein two hyphae, originating from different spores, appeared to be united by a short hyphal strand. Such an appearance could occur by the chance juxtaposition of hyphae, however, without the occurrence of fusion. The union of separate hyphae by two or more strands in close proximity was also observed and considered less likely to represent a chance occurrence. Figs. 1, 2, and 4 each show two hyphae, originating from different spores, joined by two or more hyphal strands, while Fig. 5 shows two hyphae, originating from the same spore, bridged by at least three strands.

The tannic acid – crystal violet stain for membranes provided a further test as to whether fusion had actually occurred between hyphae. This procedure stained the hyphae only lightly and did not render them opaque. Hence, where one hypha lay on top of another, the stain appeared to be much darker than where two hyphae merely touched one another. Furthermore, where two hyphae came in contact without fusing, some of the tannic acid mordant precipitated and revealed the cell walls separating the two hyphae. Anastomosis, as revealed by this stain, is shown in Figs. 3, 6, and 7.

Using the criteria of multiple union between hyphae and the absence of a demonstrable cell wall or membrane between these hyphae as evidence for fusion, it was apparent that the three streptomycetes examined possessed the ability to undergo anastomosis. The two strains of *S. scabies* appeared to be subject to a greater frequency of hyphal fusions than did the unidentified species T12. It was apparent that anastomosis occurred both in the early growth of the mycelia (less than 16 hr.) and in later growth. The incidence of anastomosis was not demonstrably influenced by the composition of the culture medium.

Occurrence of Swollen Chromatinic Structures

The acid-Giemsa stain did not reveal any unusual features in the chromatinic bodies (presumably nuclei) present at the sites of anastomoses which would indicate the formation of "initial cells". Swellings in the hyphae, both terminal and intercalary, were observed at various stages of growth, however,

which resembled those described by some workers as initial cells (3, 9). These bodies stained deeply with the Giemsa stain following acid hydrolysis or contained much chromatinic material. These structures appeared to be of at least two types.

One type of structure has been identified as the residues of spores from which the hyphae originated. When streptomycete conidia were placed in a culture medium, they swelled, to give a three- to four-fold increase in volume, prior to germination. Some spores germinated with one or two hyphae (all cultures studied) or with three and rarely four hyphae (*S. scabies* A26) (Fig. 8, A–D). For the first few hours of growth, the parent spore was readily recognized. After incubation for about 12 hours, however, the parent spore became unrecognizable when the young mycelium was viewed subsequent to the acid-Giemsa stain (Fig. 9). Parallel series of slides stained by alternate methods (e.g. crystal violet) still showed the originating-spore very clearly (Fig. 10). As the mycelium developed further (about 20 hr. incubation), the parent spore once again was clearly revealed by the acid-Giemsa stain (Figs. 11 and 12). The spores now stained uniformly dark with this stain or appeared to contain several tightly packed chromatinic bodies. The parallel series of slides, stained by the alternate procedures, made the identification of these structures as spore-residues quite clear.

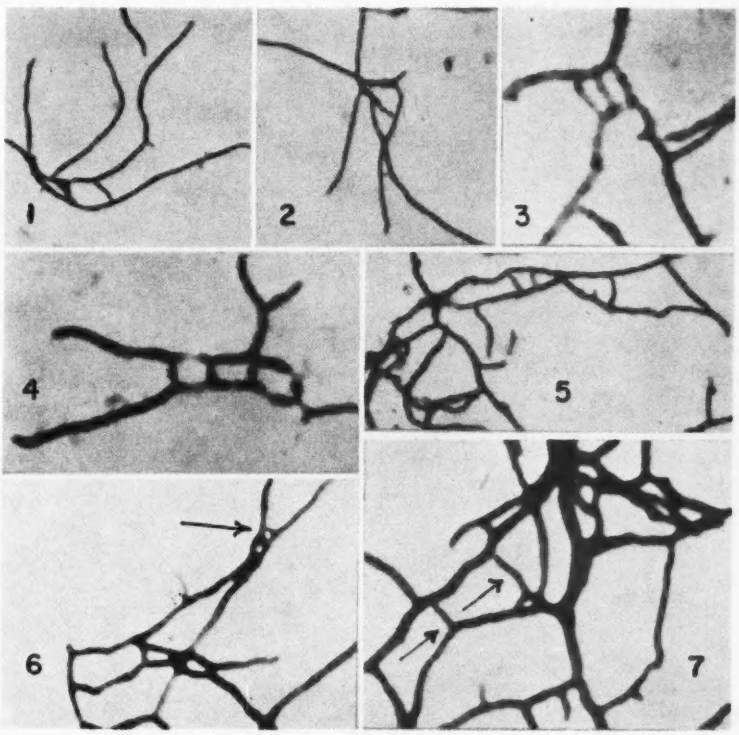
After about 44 hr. incubation, additional swollen bodies were observed. Some of these were no larger than the spore residues described above but were more nearly spherical (Fig. 13). Others were several-fold larger and irregularly shaped (Figs. 14 and 15). All of these structures observed were terminal and stained deeply, without differentiation of internal detail, with the acid-Giemsa stain. The hyphae bearing these swollen bodies likewise stained deeply without differentiation of cytological detail. These bodies appeared a few hours earlier in *Streptomyces* sp. T12 than in *S. scabies* A26 but were essentially the same in both cultures.

Occurrence of "Secondary Mycelium"

Mycelium similar to Klieneberger-Nobel's (8) "secondary mycelium" was observed in *Streptomyces* sp. T12 as previously reported by McGregor (9). The hyphae in this mycelium possessed chromatinic cylinders, more or less regularly spaced transverse septa, and fewer branches than those in the "primary" mycelium. The designations "primary" and "secondary" as used here follow the terminology of Klieneberger-Nobel (8). The identification of the mycelia is based solely on their cytological appearance. It proved to be extremely difficult to trace these secondary hyphae to their points of origin. In many cases, however, they could be traced to regions where the internal structure, as revealed by the acid-Giemsa stain, appeared to be intermediate between the primary and secondary types (Fig. 16).

The mycelium of *S. scabies* could not be differentiated clearly into primary and secondary types. Hyphae with few branches and clearly demonstrable transverse septa were observed in old cultures (Fig. 17) but these hyphae

PLATE I



FIGS. 1–7. Multiple anastomoses in species of *Streptomyces*.

FIGS. 1 and 2. Anastomoses between hyphae originating from separate spores. *S. scabies* A26, 16 hr., crystal violet stain. × 1600.

FIG. 3. *S. scabies* A26, six days, membrane stain. × 2300.

FIG. 4. Anastomoses between hyphae originating from separate spores. *Streptomyces* sp. T12, 16 hr., crystal violet stain. × 2300.

FIG. 5. Anastomoses between hyphae originating from the same spore. *S. scabies* A26, crystal violet stain. × 1600.

FIG. 6. *S. scabies* A30, 20 hr., membrane stain. × 2300.

FIG. 7. *S. scabies* A26, 20 hr., membrane stain. × 2300. Note absence of membranes at points of contact.

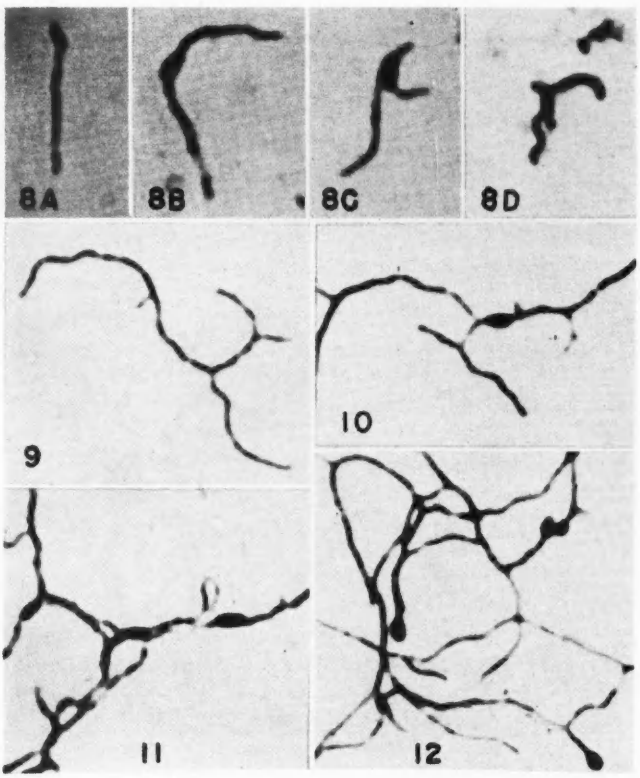


FIG. 8. Germination of conidia of *S. scabies* A26, 8 hr. × 2300. (A) One hypha. (B) Two hyphae. (C) Three hyphae. (D) Four hyphae.
FIG. 9. *S. scabies* A26, 12 hr., acid-Giemsa stain. Parent spore not recognizable. × 2300.
FIG. 10. *S. scabies* A26, 12 hr., crystal violet stain. Parent spore clearly recognizable. × 2300.
FIG. 11. *S. scabies* A26, 24 hr., acid-Giemsa stain. Parent spores deeply stained. × 2300.
FIG. 12. *Streptomyces* sp. T12, 20 hr., acid-Giemsa stain. Parent spores deeply stained. × 2300.

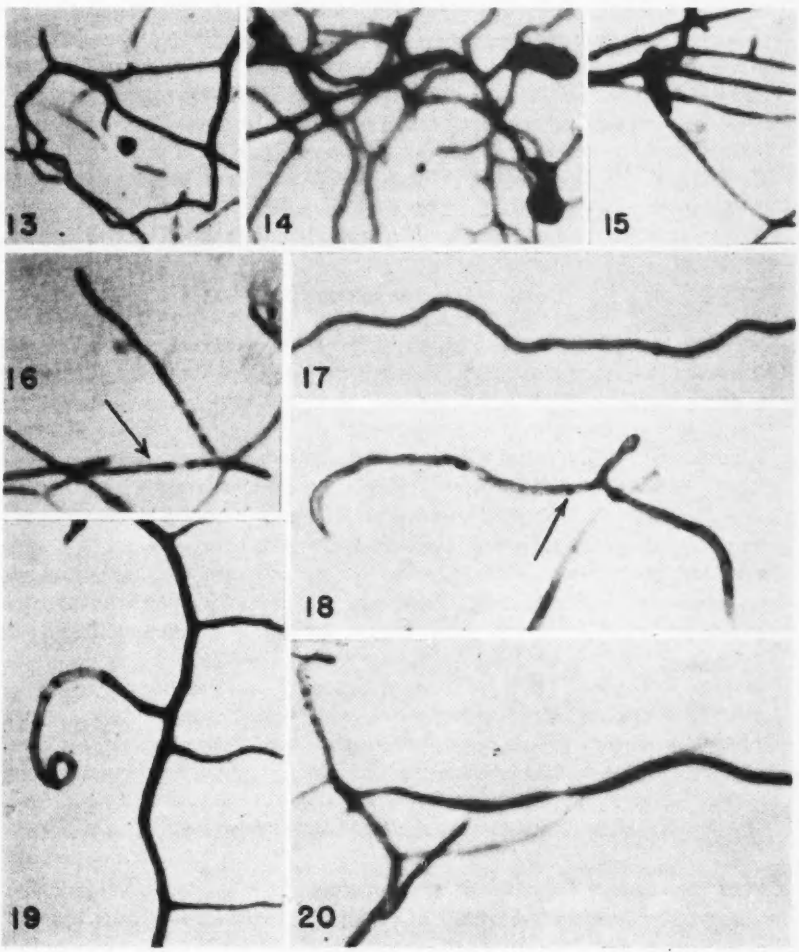


FIG. 13. *S. scabies* A26, 72 hr., acid-Giemsa stain. Unidentified swollen body. × 2300.
FIG. 14. Large irregularly shaped swollen bodies. *S. scabies* A26, 48 hr., acid-Giemsa stain. × 2300.
FIG. 15. Large swollen body. *Streptomyces* sp. T12, 48 hr., acid-Giemsa stain. × 2300.
FIG. 16. Hyphae intermediate between "primary" and "secondary" type. *Streptomyces* sp. T12, 60 hr., acid-Giemsa stain. × 2300.
FIG. 17. Septations in six-day *S. scabies* A26, membrane stain. × 2300.
FIG. 18. Septations in 16-hr. *S. scabies* A26, membrane stain. × 2300.
FIG. 19. Spore formation in *S. scabies* A26, six days, membrane stain. × 2300.
FIG. 20. Aerial hyphae originating from "primary" mycelium. *S. scabies* A26, 72 hr., acid-Giemsa stain. × 2300.

clearly originated directly from more profusely branched mycelium. Very young hyphae also often showed similarly spaced, readily demonstrable transverse septa (Fig. 18). Conidia were generally formed on short branches derived from an abundantly branched hypha (Fig. 19). The acid-Giemsa stain did show deeply stained, little-branched hyphae which contrasted with the lightly stained, branched mycelium containing discrete nuclear bodies. Many of these hyphae could readily be traced to their points of origin from hyphae resembling those described as primary (Fig. 20). No special structure or initial cell could be identified at this point of origin.

Discussion

The abundant occurrence of multiple unions, by short hyphal strands, of neighboring hyphae, together with the absence of demonstrable cell walls or membranes at the points of contact, indicate that hyphal anastomosis is of common occurrence in the three strains of streptomycetes examined. Although septa were clearly demonstrable by the tannic acid – crystal violet stain, the cells were multinucleate. Hyphal fusion in these organisms could, therefore, readily produce a heterokaryotic condition.

Erikson (4) has suggested that the dark-staining discoid structures revealed by the tannic acid mordanting technique may not be true septa but may result from the action of tannic acid as a protein precipitant. The coherence of the streptomycete mycelium, as compared to the ready rupturing of the mycelium of the nocardias, where septa may be observed without prior mordanting, was offered in further support of this view. The regular occurrence of these structures, in many hyphae of the strains examined, and their similarity to the undisputed septa formed during sporulation, make this interpretation less likely. It must be remembered that although septation may be followed by fragmentation of the mycelium among some of the related members of the Actinomycetales, many of the septate molds maintain a firmly coherent mycelium.

The failure to note any nuclear changes at the sites of anastomoses does not, in itself, mean that such might not have occurred if further development had been permitted. The formation of large swollen initial cells as the result of anastomosis appears to be improbable in these strains, however, since none of the observed structures fulfilled the necessary criteria. The parent spores are persistent and have the peculiar property of not containing chromatinic material during the early development of the hyphae but of staining deeply with the acid-Giemsa stain at a later stage. If a spore had germinated with one hypha only, it would subsequently appear to be on the end of a stalk, whereas if it had germinated at two points, it would appear to be in the middle of a hypha. If one were to examine various ages of streptomycete mycelium by the acid-Giemsa stain alone, these spore-residues might seem to appear de novo. Hence, care should be taken not to interpret these structures as initial cells.

The other swollen chromatinic structures observed were invariably terminal. These could not, therefore, have arisen at the sites of anastomoses. If their formation were related to hyphal fusion, then a considerable amount of growth must have occurred following the anastomoses and prior to the formation of the swollen bodies. In all cases, these structures developed concurrently with, or subsequent to, the development of hyphae cytologically characteristic of the secondary mycelium. It appears to be unlikely, therefore, that the secondary mycelium arose from them. The hyphae on which these bodies were borne were undifferentiated and not typical of either primary or secondary types. The function of these swollen bodies, if any, was not apparent. Enlarged structures have also been described in *S. griseus* (12). Erikson (5) observed many large swollen cells in *S. scabies* and *S. coelicolor* grown on media containing a detergent or pectin. She considers that the bloated shapes frequently encountered in actinomycetes result from overfeeding and other unfavorable conditions.

Klieneberger-Nobel (8) considered that the structure of the secondary mycelium favored the assumption that a fusion cell with a fusion nucleus initiated its formation. Since hyphae apparently representative of secondary mycelium in *S. scabies* A26 may originate directly from typical primary mycelium (Fig. 20), initial cell formation is clearly not a necessary prerequisite for the development of secondary or aerial hyphae. Evidence for the direct development of secondary mycelium from primary mycelium in *Streptomyces* sp. T12 is less conclusive. The existence of hyphae with an appearance intermediate between these two types, however, suggests that, here too, no fusion cell or fusion nucleus need precede the formation of mycelium cytologically identifiable as secondary mycelium. These observations suggest, therefore, that the development of "secondary" mycelium in the strains of streptomycetes studied results from physiological changes rather than an alternation of haploid and diploid phases as suggested by Klieneberger-Nobel (8). These changes could be associated with exhaustion of nutrients, maturity of the mycelium, or some other factor. Newcombe (11) noted one instance in which the environment affected the cytological appearance of the nuclei in a streptomycete. Intermittent incubation (alternating with chilling) resulted in nuclei being clearly visible in a lightly stained cytoplasm, whereas with continuous incubation the stainable material nearly filled the cell.

The rarity of genetic recombination in conidia formed by heterokaryons of streptomycetes (1, 16) agrees with the conclusions presented here. If conidia were formed by meiosis from diploid nuclei of secondary mycelium, one would anticipate a much greater incidence of recombined characters in the haploid conidia than has been observed.

Acknowledgment

This work was assisted by a grant from the National Research Council of Canada whose support is gratefully acknowledged.

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STUDIES ON BACTERIAL AGGLUTINATION BY USE OF THE ANTIGLOBULIN (COOMBS) TECHNIQUE[1]

By Arthur C. Ford and Ralph J. DeFalco

Abstract

The Coombs antiglobulin test has been successfully applied to bacterial cells in agglutination reactions. The test is a test for attached antibody globulin. Titers obtained by this method are shown to be from one to three tubes higher than those obtained by routine agglutination tests. Polyvinylpyrrolidone (PVP), bovine albumin, and human albumin have also been shown to hasten and strengthen bacterial agglutinations. The incomplete albumin reacting antibody titer of antisera seems to be higher than that of the saline reacting, or complete antibodies. The Coombs reaction is a specific test for antibody globulin attached to sensitized cells. There is no reaction where there is no combination between antibody globulin and the cell antigen, as shown by heterologous tests.

It has been shown by Coombs and co-workers (1) that Rh antibodies occur in two forms. One, the saline type of agglutinin, gives direct reactions with saline-suspended cells. The other is an albumin type of agglutinin in which, although specifically combining with saline-suspended cells, fails to produce visible agglutination. The latter is the "blocker" type of antibody. The blocker antibodies will, however, give direct reactions with Rh positive cells suspended in bovine albumin (3), and such agents as polyvinylpyrrolidone (PVP) (10). Blockers also give direct reactions with trypsinized cells suspended in saline. The albumin type of antibody has been shown to be present in antisera other than anti-Rh. In fact, they are probably present in all antisera.

The antiglobulin test was first described by Moreschi (12), who applied these principles to the agglutination of rabbit cells which had been sensitized with goat antibody. He reported that the agglutination of these cells was considerably enhanced by the use of an antigoat globulin serum. Later in the same year (13) he described the acceleration and strengthening of bacterial agglutinations by a similar technique.

Since the rediscovery of the technique by Coombs *et al.*, several investigators have applied it to bacterial agglutinations. It has been used to show increased titers in the serum of patients with specific bacterial infections as well as to detect natural agglutinins in human sera against certain bacteria (6). Among these infections were: typhoid (14, 17, 5, 6), *Shigella* (14), Q-fever (2), *Brucella* (8, 16, 4, 19, 20), and tuberculosis (11, 15, 18). In these tests, the titers obtained by use of the antiglobulin technique were compared with those obtained in saline media. In most cases the titer was from one to three tubes higher when the antiglobulin test was used. Gabrielli *et al.* (5) reported that incomplete antibodies were demonstrated by the Coombs method in many cases of typhoid fever where agglutination was not apparent in saline. Wagner and Kuhns (19) reported the same phenomenon in antibrucella sera.

[1]*Manuscript received July 3, 1956.*
Contribution from the Department of Bacteriology and The Serological Museum, Bureau of Biological Research, Rutgers University, New Brunswick, New Jersey, U.S.A.

The present paper deals with applications of the Coombs test to bacterial agglutinations using rabbit antisera and several bacterial species. The use of PVP and albumin in acclerating and strengthening bacterial agglutinations is reported, we believe, for the first time.

Materials and Methods

Preparation of Antigens

The following organisms were used as antigens: *Salmonella typhosa*, *Salmonella schotmuelleri*, *Salmonella paratyphi*, *Shigella shiga*, *Pseudomonas fluorescens*, *Escherichia coli*, *Aerobacter aerogenes*, *Diplococcus pneumoniae* type II, and *Proteus vulgaris*.

Twenty-four-hour cultures of the organisms were grown on brain heart infusion agar slants in large, flat flasks at 37° C. Growths were harvested from the flasks by covering the surface with sterile 0.85% saline and suspending the organisms with a sterile loop. The cells were washed twice in sterile, buffered (pH 7.2) saline. After the last wash, the organisms were resuspended in formolized (0.2 %) saline. The antigens were diluted so as to contain approximately 900×10^6 organisms per ml. The finished antigens were stored at 4° C. until used.

Preparation of the Antisera

The antisera were prepared in rabbits. Antigens were administered in 0.5 ml. amounts daily in a series of three subcutaneous injections. Sample bleedings were taken on the sixth day following the last injection. Final bleedings were made on the seventh day if a satisfactory agglutinin titer had been produced. The rabbits were exsanguinated by cardiac puncture. The sera were collected and placed in sterile bottles to which merthiolate was added to a final concentration of 1 : 10,000.

Antirabbit serum was prepared in chickens. This was accomplished by injections of whole rabbit serum. The methods of injection, collection, and preparation of this serum were the same as those used in preparing the antisera in the rabbits.

The prepared antisera were stored at 4° C. until used.

Tests for Titers

The bacterial agglutination test was used to determine the titer of the antisera prepared in the rabbits. Equal amounts of increasing dilutions of the antisera were placed in Kahn tubes to which was added an equal volume of antigen suspension. Readings were taken after two hours' incubation at 37° C. and also after overnight incubation in the refrigerator. The recorded titer was the last tube in which agglutination of the bacterial cells had occurred.

The precipitin reaction was used to determine the titer of the antiserum which was prepared in the chicken. The photronreflectometer (Libby, 1938) was used to ascertain the range of reaction between the antigen and the

antiserum. If the readings were satisfactory, the antiserum was diluted 1 : 20 and stored at 4° C. This serum served as the Coombs reagent in the tests which will be described.

Performance of the Coombs Test on Bacterial Cells

The first part of this test is identical with the bacterial agglutination test previously described. The tubes were then incubated at 37° C. for 20 min. At the end of this time they were centrifuged at moderate speed for three minutes. The supernates were discarded. The cells were washed three times with buffered saline (0.85%). Following the last washing, the cells were resuspended in 0.2 ml. of 0.85 % saline. To each suspension was added 0.2 ml. of a 1 : 20 dilution of antirabbit serum (the Coombs reagent). The tubes were incubated for an additional 20 min. at 37°C. At the end of this period the tubes were gently flicked with the fingers in order to resuspend the cells. They were then centrifuged in the clinical centrifuge at moderate speed for one minute. The tubes were again agitated to resuspend the cells. The titer was recorded as the last tube showing agglutination. In some tests PVP, bovine albumin, and human albumin were substituted for the antirabbit serum.

Routine agglutination tests were run simultaneously using the same antigen–antibody systems and the same dilution series. The routine tests were incubated in the refrigerator overnight following one hour's incubation at 37° C. On the following day the titers were recorded and compared with those obtained by the Coombs method.

Experimental Results

Homologous Reactions

Preliminary work consisted of doing Coombs and routine agglutination tests on all of the homologous systems selected for investigation. The purpose of these tests was to determine if the Coombs test could be applied to all of these systems and to compare the titers obtained with those of the routine tests. The results indicated that the antiglobulin test could be

TABLE I

COMPARATIVE TITERS WITH AND WITHOUT COOMBS AFTER ANTIGEN–ANTIBODY HAS REACTED FOR 20 MIN.

System	Titers	
	Routine	Coombs
typhosa × anti-*typhosa*	1:2560	1:5120
coli × anti-*coli*	1:128	1:256
Pseudomonas × anti-*Pseudomonas*	1:512	1:1024
aerogenes × anti-*aerogenes*	1:32	1:64
Proteus × anti-*Proteus*	1:1280	1:2560
Shigella × anti-*Shigella*	1:128	1:512
Pneumococcus × anti-*Pneumococcus*	1:320	1:640

applied to all of the systems, and that the titers obtained by this method are from one to two tubes higher than those obtained by the routine method. The results are shown in Table I.

Increase in Overnight Titers as Shown by the Coombs Method

The next concern was to determine if the titers obtained by overnight incubation of the routine tests could be shown to be higher by the addition of antirabbit serum to the tubes in which no agglutination was apparent. The cells in those tubes were washed three times in buffered saline. The cells were then resuspended in buffered saline (0.2 ml.). To each tube was added 0.2 ml. antirabbit serum. The tubes were incubated at 37° C. for 30 min., centrifuged, and checked for agglutination. The titers were shown to be increased by from one to three tubes. The results of several such tests are shown in Table II.

TABLE II

COMPARATIVE TITERS WITH AND WITHOUT COOMBS AFTER OVERNIGHT INCUBATION OF ANTIGEN–ANTIBODY MIXTURE

System	Routine overnight titer	Coombs titer
Proteus × anti-*Proteus*	1:1280	1:5120
typhosa × anti-*typhosa*	1:2560	1:10240
coli × anti-*coli*	1:128	1:512
Pseudomonas × anti-*Pseudomonas*	1:512	1:2048

The Effects of PVP, Bovine Albumin, and Human Albumin on Bacterial Agglutinations

As has been noted, polyvinylpyrrolidone (PVP), albumin, and other non-specific substances of high molecular weight have been shown to be of considerable value in accelerating and strengthening the reaction between Rh positive cells and anti-Rh serum. Three of these substances were used in this work. PVP, bovine albumin, and human albumin were chosen. Four antigen–antibody systems were used to demonstrate the effects of these substances on sensitized bacterial cells. Routine and modified Coombs tests were performed as previously described. After the third washing the cells were resuspended in 0.2 ml. buffered saline. Six tubes of each antigen–antibody dilution series were prepared and the reagents were added in the following manner:

Row 1. None, incubated 24 hr. at 37° C.
Row 2. Antirabbit, 0.2 ml.—true Coombs.
Row 3. Ten per cent PVP (0.2 ml.).
Row 4. Ten per cent PVP plus antirabbit serum (0.1 ml. of each)—modified Coombs.
Row 5. Twenty per cent bovine albumin (0.2 ml.).
Row 6. Concentrated human albumin (one drop).

After 20 min. incubation at 37° C., the tubes were centrifuged for one minute and observed for clumping. It was shown that these substances could be used in bacterial agglutinations with the results obtained being as found in the Rh tests. As shown in Table III, the results compare very well with those obtained by the use of the antirabbit serum, and the titers are higher than those shown by the routine tests.

TABLE III

THE EFFECTS OF PVP, BOVINE ALBUMIN, AND HUMAN ALBUMIN ON BACTERIAL AGGLUTINATIONS

System	Routine	Coombs	PVP	Titers Coombs+ PVP	Bovine albumin	Human albumin
coli × anti-*coli*	1:640	1:2560	1:2560	1:2560	1:2560	1:2560
typhosa × anti-*typhosa*	1:1280	1:5120	1:5120	1:5120	1:5120	1:5120
Proteus × anti-*Proteus*	1:1280	1:5120	1:5120	1:5120	1:5120	1:5120
Pseudomonas × anti-*Pseudomonas*	1:640	1:2560	1:2560	1:2560	1:2560	1:2560

Controls

1. A series of controls using normal rabbit serum were set up. Both Coombs and routine tests were performed. All such tests were negative. This indicated that the rabbits used had no natural agglutinins for the bacterial cells which were used in the tests. The possibility of non-specific results was also ruled out.

2. Another set of controls consisted of using unsensitized bacterial cells and the antirabbit serum. All were negative, indicating that the antirabbit serum was specific for antibody globulin when used in the Coombs test.

3. PVP, bovine albumin, and human albumin were added to suspensions of unsensitized cells to determine the specificity of their action. Again all of the tests were negative.

Heterologous Tests

Coombs and routine agglutination tests were performed on heterologous antigen–antibody systems in order to determine the specificity of the reactions and the validity of the results obtained in the homologous tests. As shown in Table IV, there were no reactions in the absence of established serological relationships. The systems reacting do show an increase in titer of two tubes following overnight incubation and Coombs tests.

PVP, Bovine Albumin, and Human Albumin in Heterologous Tests

Our results indicated that only the antirabbit serum gives a higher titer in heterologous Coombs tests. The other substances only enable one to obtain the titers more rapidly. PVP, when combined with antirabbit serum, does show a higher titer. This is probably due, however, to the specific

TABLE IV

COMPARATIVE COOMBS AND ROUTINE TITERS IN HETEROLOGOUS TESTS

System	Routine	Titers Coombs	Coombs overnight
Anti-*typhosa* × *S. paratyphi*	1:64	1:128	1:256
Anti-*typhosa* × *S. schottmuelleri*	1:32	1:64	1:128
Anti-*typhosa* × *E. coli*	—	—	—
Anti-*coli* × *proteus*	—	—	—
Anti-*coli* × *pseudomonas*	—	—	—
Anti-*proteus* × *A. aerogenes*	—	—	—
Anti-*Shiga* × *A. aerogenes*	—	—	—

reaction between the globulin of the rabbit serum absorbed on the cells and the antiglobulin fraction of the antirabbit serum—the PVP merely making a stronger and more clear-cut reaction.

Discussion

It can be stated that, in bacterial agglutinations, the Coombs test almost always results in higher titers being obtained. It can be further stated that the test offers a more rapid means of obtaining titers.

It was not the purpose of this investigation to elucidate the mechanism of the Coombs test. It would seem, however, that the reaction occurring when the antirabbit serum is used is a manifestation of a specific combination between an antigen (specific bacterial agglutinin) and an antibody (antirabbit globulin). Specific groups on the antibody molecule combine with the determinant or complementary groups of the antigen. Thus when the rabbit globulin is adsorbed on the surface of the bacterial cells, certain groups which are capable of reacting with the antirabbit globulin (Coombs reagent) are left exposed. Addition of the antirabbit serum is followed by specific combination between these groups. This is followed by aggregation and agglutination.

But what about the reaction occurring when such non-specific substances as PVP and albumin are added? In order to answer such a question, we must consider the types of antibody found in antisera. It is generally accepted that each antiserum contains more than one type of antibody. Some of these are the "complete" or "saline-type" antibodies. Others are the "incomplete" or "albumin" type. The latter are shown only by special techniques such as the Coombs test and are present in higher concentration than the former. They may be formed as a response to antigens which have undergone partial disintegration in the body or they may be what Haurowitz (7) calls poorly-adapted antibodies. These antibodies are described by Haurowitz as antibodies which are imperfectly adapted to the antigen molecule. He believes that the multiplicity of antibodies in an antiserum is due not only to the fact that they are complementarily adapted to different

portions of the antigen molecule, but also to their imperfect adaption. Since antibody molecules are made up of amino acids and since the peptide chains cannot be distorted beyond a certain limit, Haurowitz believes that the complementary adaptation of the antibody molecule to the antigen molecule will approach perfection but will never really be perfect. In other words, the template does not elicit the formation of a perfect replica. The farther away it is from perfection, the poorer the antibody adaptation.

We might assume, then, that the antibody titer which is obtained in saline media is due mainly to the well-adapted or near-perfect antibody molecules of the antiserum. These would be the "complete" antibodies. On the other hand, the poorly-adapted or "incomplete" antibody molecules do not show agglutination in saline. Something else is needed for their detection; in effect, a sensitizer for the sensitizer. The antirabbit serum and the non-specific substances such as PVP, bovine albumin, and human albumin supply the factor or factors needed for aggregation. The non-specific substances supply, in some manner, the complementary surface needed. It is known that the union between the antirabbit serum and the rabbit antibody on the cells is a much stronger one than that obtained with the non-specific substances. These substances *may* form a looser combination because they only serve to "cement" the poorly adapted antibody and the antigen molecule together, most probably through alterations in the surface charges.

If we accept the hypothesis that each antiserum is composed of both types of antibody in varying concentrations, it can be readily seen that the only way of obtaining the true antibody content of an antiserum is to perform tests for both types of antibody. These tests may be very useful in the diagnosis of certain diseases where the "complete" antibody titer is very low or non-existent. Such diseases as those caused by animal parasites, allergens, fungi, and viruses may possibly be detected with much more facility by applications of the Coombs test or by modifications of this technique. We believe that a wider application of this technique in serological tests may be well worth the effort.

Acknowledgments

The authors are indebted to Professor T. J. Murray of the Department of Bacteriology for the bacterial cultures used in this work.

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EXTRACELLULAR POLYSACCHARIDES OF GREEN ALGAE[1]

By Ralph A. Lewin[2]

Abstract

Eighteen unicellular or colonial green algae, mostly *Chlamydomonas* species, were isolated from soil or fresh water and grown in mass culture. All of these species liberated some soluble polysaccharides into the growth medium. This material constituted 25% of the total organic matter produced by the cells of *Chlamydomonas mexicana*. Comparable quantities were isolated from other species by extraction of the cells with hot water or dilute alkali.

In all species but one, galactose and arabinose were the main components of the polysaccharides. In *Chlamydomonas ulvaënsis*, glucose and xylose predominated. Associated sugar moieties included fucose, rhamnose, mannose, uronic acids, and several unidentified components. These constituents were separated and identified by paper chromatography.

The role of such polysaccharides in the ecology of soil and natural waters is discussed.

Introduction

The cells of certain green algae characteristically form mucilaginous capsules, presumed to be polysaccharide in nature. In addition, it has been observed that many species liberate soluble organic materials into the medium. In the studies described here, attention was centered on a preliminary characterization of the extracellular soluble and capsular polysaccharides formed by 18 selected species. No account was taken of the production of free sugars or organic acids, which have recently received attention from Allen (1), or of soluble nitrogenous materials, which have been investigated by Fogg and Westlake (6).

Materials and Methods

(*a*) *Isolation and Culture of Algae*

Most of the strains investigated were isolated from soil samples, collected in a variety of climates: the rest were obtained from wet sand or mud at the borders of lakes or streams. They were grown in a basal medium (MM) containing (w/v) $Ca(NO_3)_2 \cdot 4H_2O$, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.02%; K_2HPO_4, 0.02%; and traces of Fe, etc. Enrichment cultures in this medium were illuminated for two to three weeks, and then streaked on agar plates (MM + 1% agar) and again illuminated for a similar period. Clones which formed mucilaginous colonies were selected, subcultured in liquid medium, and freed from bacteria by restreaking on agar. Three non-mucilaginous species, isolated from similar habitats, were included for the purposes of comparison. Stocks were maintained on slants of the basal medium supplemented with 0.2% Bacto-tryptone (Difco). Mass cultures were grown at 23° in 2-liter flasks containing 1.5 liters of medium (MM), constantly illuminated under

[1]*Manuscript received July 30, 1956.*
Contribution from Atlantic Regional Laboratory, National Research Council, Halifax, N.S., Canada. Issued as N.R.C. No. 4119. A preliminary report appeared in Phycological News Bulletin, 8 : 6. 1955.
[2]*Present address: Marine Biological Laboratory, Woods Hole, Mass., U.S.A.*

white fluorescent lights at an intensity of 450 ft-c. The medium was supplemented with thiamine (1 mgm./liter) for the growth of *Gloeocystis gigas*, and with cobalamin (1 μgm./liter) for that of *Chlorosarcina consociata*. Aeration and turbulence were provided by a constant flow of sterile air.

(*b*) *Extraction of Polysaccharides*

After two weeks of growth the cells were separated by centrifugation, and the supernatant clarified by filtration. The filtrate was concentrated to 40 ml. by being boiled in an open dish in a forced draught, and, after being cooled, was poured into 120 ml. of 95% ethanol. Extracellular polysaccharide, when present, appeared as a flocculent or stringy precipitate. It was collected by centrifugation, washed with ethanol, dried at room temperature first in an air draught and then overnight in a desiccator over concentrated sulphuric acid, weighed, and pulverized. This material frequently contained an appreciable fraction of mineral matter, which was determined quantitatively from the weight of the ash residue after a 10 mgm. sample was incinerated in a platinum crucible. The yield of crude ash-free polysaccharide (fraction S) was then calculated.

The cells were killed with 0.2 ml. of a mixture of 1 part chlorobenzene: 1 dichlorethane: 2 chlorobutane (9), and extracted with 50 ml. water at 5° for two days (fraction C-1), then with 50 ml. water at 120° in the autoclave for 15 min. (fraction C-2), and finally with 50 ml. 1% NaOH at 120° for 15 min. (fraction C-3).

Fractions C-1 and C-2 were concentrated to 10 ml. and poured into 30 ml. of 95% ethanol. When a precipitate formed, it was removed, washed, and dried as described above. Fraction C-3 was acidified with hydrochloric acid to pH 2.0, and the precipitate, containing degraded chlorophylls, proteins, etc., was removed by centrifugation (fraction C-3*a*). The supernatant was evaporated to 10 ml. and poured into 30 ml. of 95% ethanol, to give fraction C-3*b*. Finally, the extracted cell residues were washed, dried, and weighed. The values given in Table I, under "cell residues", have been adjusted to include the fraction C-3*a*.

(*c*) *Analysis of Polysaccharides*

Ten-milligram samples were hydrolyzed with 1.0 ml. of 0.7% H_2SO_4 in sealed glass ampoules for six hours at 105°. The acid was then neutralized with barium carbonate, the precipitate aggregated by boiling and removed by centrifugation, and the supernatant concentrated by evaporation to 0.1 ml. The products of hydrolysis, along with known sugars for reference, were examined by chromatography on Whatman No. 1 paper. The solvent generally used was a mixture of *n*-butanol, water, and acetic acid (4 : 4 : 1, upper layer). Descending chromatograms were run at room temperature for 16–20 hr., with migration of the solvent front approximately 30–40 cm. Glucose and galactose, which exhibit similar R_f values in this solvent, were

clearly distinguished by the use of a solvent mixture of phenol and water (4 : 1). The chromatograms were dried, sprayed with 2% aniline phthalate in 95% butanol, and developed at 105° for four minutes. Spots indicating pentoses and uronic acids were rosy, those indicating hexoses and methylpentoses were grayish-brown.

The amounts of polysaccharides in solution were determined by a modification of the method of Dreywood (5). Four milliliters of freshly prepared reagent (0.2% anthrone in 95% H_2SO_4) at room temperature was mixed rapidly with 2.0 ml. of a solution in a 30 ml. test tube, and allowed to stand for 10 min. The value for light absorption of the mixture, determined in a Klett colorimeter with a green (540 mμ) filter and corrected for control, was found to be proportional to the concentration of glucose within the range 0–100 p.p.m. Determinations were made after suitable dilutions, and soluble polysaccharide was expressed as its glucose-equivalent.

Results

(*a*) *Identification of Algal Isolates*

Cells from young, active cultures in liquid medium were examined, and in most cases could be readily assigned to the genus *Chlamydomonas*. Gerloff (7) recognized 321 species of this genus, and at least 100 more have already been described; exact identification of species of *Chlamydomonas* is therefore difficult. Three strains which in certain particulars did not conform with any of the species so far described are designated with a provisional specific name; Latin diagnoses are to be published elsewhere. All identifications should be regarded as tentative.

(*b*) *Yields of Polysaccharides*

The results are summarized in Table I. Total dry weights of organic matter recovered, i.e. cells plus polysaccharide, were of the order of 0.5 gm./liter. Soluble polysaccharide liberated into the medium by *Chlamydomonas mexicana* ranged as high as 0.1 gm./liter, which represents 20–25% of the total organic matter produced. The diffluent mucilage around cells of *C. parvula* and *C. peterfii* under these conditions represented 40–60% of the total organic matter, and in old cultures was produced in such quantities that the viscosity of the medium was appreciably increased. The mucilaginous capsule of the palmelloid *Gloeocystis gigas* swelled and dissolved when heated in water, but set to a gel again on cooling. The thick walls of *C. callosa* were more soluble in hot alkaline solution than at neutral pH; they represented about 30% of the total dry matter. In some cases, digestion with hot 1% NaOH resulted in the complete disruption and solution of the cell walls, whereas in others these remained intact after such treatment. Clear distinctions cannot be readily drawn between soluble, liberated polysaccharide and diffluent sheath, between the latter and a firm capsule, or between a capsule and the cell wall itself.

TABLE I

YIELDS (MGM. DRY WEIGHT/1.5 LITERS) OF POLYSACCHARIDES AND CELL RESIDUES FROM TWO-WEEK CULTURES OF VARIOUS UNICELLULAR ALGAE; AND CONSTITUENT SUGAR MOIETIES OF THE POLYSACCHARIDES ISOLATED

Genus and species	Source*	Polysaccharide in extract, mgm. S	C-1	C-2	C-3*b*	Total polysacch., mgm.	Cell residue, mgm.	Total organic matter, mgm.	% polysacch.	Constituent mono-saccharides†
I. Non-mucilaginous species										
Chlamydomonas dysosmos Moewus	12	24				24	533	557	4	AG
" *moewusii* Gerloff	3	43				43	514	557	8	AG
" *philotes* nom. prov.	4	14	17	6		37	451	488	8	AG
II. Mucilaginous species										
Chlamydomonas acidophila Negoro	13	55				55	305	360	15	AG
" *applanata* Pringsheim	2	106		89		195	310	505	39	AG-r
" *callosa* Gerloff (*a*)	13	4	16	73	176	269	497	766	35	AGR-fz
" " (*a*)	8	55	35	40		130	351	481	27	AGR-fz
" " (*b*)	11	48	22	31	215	316	367	683	46	AGU-fr
" *debaryana* Goroschankin	7	36	37	85		158	629	787	20	AGRU
" *inflexa* Pringsheim	6	61	14	46		121	514	635	19	AGMR-fz
" aff. *inflexa* (TT. 1/19)	2	27		54	181	262	706	968	27	AG-mfrx
" *mexicana* nom. prov.	10	141	14	86		241	471	712	34	AFG-dr
" "	7	170		47		217	438	655	33	AFG-dr
" *parvula* Gerloff	13	51	15	155		221	318	539	41	AG
" *peterfii* Gerloff	5	53		391		444	335	779	57	AG
" *sphagnophila* Pascher	9	27	19	22	255	323	358	681	47	AG-mrxyz
" *ultrafusis* nom. prov.	13	73	120	143	158	494	650	1144	43	DX-az
" sp. (B.5)	1	124		20		144	262	406	35	AG
Chlorosarcina consociata (Krebs) Smith	9	18	7	28	159	212	597	809	26	AG-frz
" sp. (DD.1/26)	13	29	4	13	106	152	326	478	32	AGU-y
Gloeocystis gigas (Kütz.) Lagerh.	9	33	12		36	81	324	405	20	AG-fr
" " "	13	52	8		62	122	430	552	22	AG-r

Key to localities:

1. *Baffin Island, Canada.*
2. *Bermuda.*
3. *Chicago, Ill., U.S.A.*
4. *Chihuahua City, Mexico.*
5. *Devon Island, Canada.*
6. *Glasgow, Scotland.*
7. *Madera, Chihuahua, Mexico.*
8. *Madurodam (Hague), Netherlands.*
9. *Isle of Mull, Scotland.*
10. *Navajo, Arizona, U.S.A.*
11. *Shannon, Ireland.*
12. *Staten Island, N.Y., U.S.A.*
13. *Ulva Isle, Scotland.*

†*Key to monosaccharides:*

A. Arabinose
D. Glucose
F. Fucose
G. Galactose
M. Mannose
R. Rhamnose
U. Uronic acid
X. Xylose
Y. Unknown
Z. Unknown

Trace amounts indicated by lower-case letters.

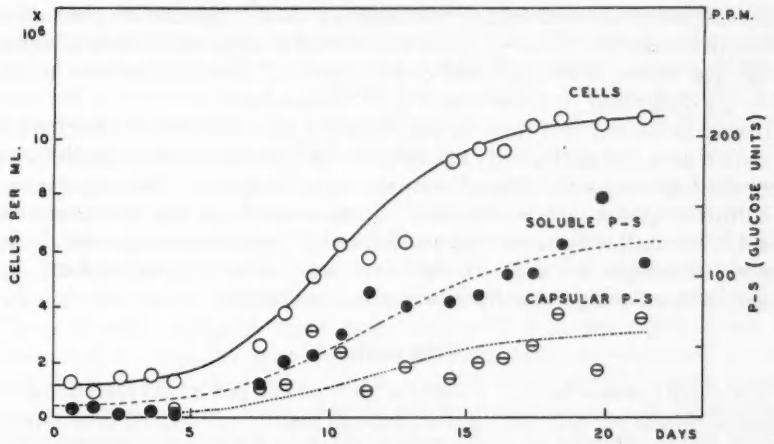

FIG. 1. Cell multiplication and polysaccharide production in *Chlamydomonas parvula*.

The course of production of polysaccharide by *C. parvula* was followed by removing 10 ml. samples at intervals from a 1.5 liter culture. The free, soluble polysaccharide in the supernatant from an aliquot was determined with the anthrone reagent. Another aliquot was heated for five minutes in a boiling water bath, a drop was removed for the determination of cell numbers, and in the remainder, freed from cells by centrifugation, the total soluble polysaccharide was determined. The amount of capsular polysaccharide soluble in hot water was calculated by difference. The results are shown in Fig. 1.

(*c*) *Chemical Composition of Polysaccharides*

Although the different fractions from each algal strain were hydrolyzed and examined separately, it was found as a general rule that, within the clone, qualitative differences of composition were minor or absent. Slight variations were observed in a few cases, e.g. the presence of an additional slow-moving component in the polyuronide range, but these have been omitted from Table I.

All but one of the polysaccharide samples isolated were made up largely of galactose and arabinose. That of *Chlamydomonas ulvaënsis* was unique in that its component sugars were glucose and xylose. (The glucose found in hydrolyzates of fractions C-2 and C-3*b* from several other species probably originated from starch which may have dissolved from the cells.) Fucose and rhamnose occurred, in many cases together, in about half of the species examined. The presence of fucose is of interest, since its occurrence in microbial polysaccharides is rare (24), and it has not hitherto been reported in a green alga. Mannose occurred in the polysaccharides of *C. inflexa* and *C. sphagnophila*. Two or three slow-moving sugars or uronic acids were present in many of the hydrolyzates examined, but were not characterized further. In certain chromatograms, spots occurred at other positions,

indicating the presence of unidentified sugars, possibly degradation products of excessive hydrolysis. The R_f values were 0.28, falling between those of xylose (0.22) and fucose (0.30), and 0.45 or 0.59, beyond that of rhamnose (0.37). They are designated in Table I as *Y* and *Z* respectively.

It may be pointed out that, although cells of the three strains identified as *C. callosa* were morphologically similar, the composition of the polysaccharide from the Irish strain (*b*) differed from that of strains (*a*). This suggests that two distinct species may be involved. In the case of the alga from Bermuda (TT.1/19), subtle differences from the Scottish *C. inflexa* were apparent during growth, although the cells of the two isolates were very similar; the dissimilarity of the polysaccharides was not unexpected.

Discussion

The results shown in Fig. 1 indicate that production of polysaccharide in *Chlamydomonas parvula* cultures parallels cell multiplication, and thus appears to be a concomitant of growth. Essentially the same result was obtained by Allen (1) with another species of *Chlamydomonas*. In this respect these algae differ from the diatom *Navicula pelliculosa*, in which capsular polysaccharide is accumulated under conditions where photosynthesis continues while cell division is inhibited by a deficiency of some essential mineral nutrient (15).

Though the chemical nature of many of the polysaccharides of the larger red and brown algae is known (20), few studies have been made on representatives of other algal classes. Hough, Jones, and Wadman (8) and Bishop, Adams, and Hughes (3) have chemically characterized the polysaccharide slime-sheaths produced by certain Cyanophyceae. Miwa (19) isolated from *Enteromorpha* sp. a hemicellulose containing rhamnose units. Kylin (13) extracted pectic materials, which he called ulvin and ulvacin, from *Ulva lactuca* and *Enteromorpha intestinalis*, and detected a methyl pentose (possibly again rhamnose ?) in the hydrolyzate of ulvin. Percival (21) isolated from *Cladophora rupestris* a sulphated polysaccharide which appeared to comprise arabinose, galactose, xylose, rhamnose, and glucose residues. Kinzel (11) obtained indications from staining experiments that the mucilages of many fresh-water algae, including Chlorophyceae such as *Gloeocystis ampla*, are acidic in character. Preliminary studies of the polysaccharides of two marine flagellates, *Chlamydomonas euryale* and *Platymonas subcordiformis*, have been reported (16). As yet there has been no detailed study of the composition and molecular structure of any mucilage from a unicellular green alga.

Ecological Implications

The occurrence of free organic solutes in natural waters has received attention from many ecologists, and there has been considerable speculation on the roles of such substances in nature. The subject has been reviewed by Krogh (12) and Lucas (18), and more recently by Fogg and Westlake (6) and Johnston (10). However, the chemical composition and biological origins of soluble organic matter liberated into lakes or oceans have remained largely

unknown. It undoubtedly comprises free organic acids, polypeptides, sugars, and polysaccharides, often in appreciable quantities. For example, Wangersky (23) isolated in crystalline form a rhamnoside from inshore waters, where it occurred in concentrations as high as 100 mgm./liter. To some extent such substances derive from the excretory products of animals and from the autolysis and decay of other living matter.

However, it has been postulated that among the sources of organic solutes are the cells of living phytoplankton, which, even in the intact state, may be seen to liberate colloidal material in the form of diffluent mucilaginous sheaths. The results presented here provide details in support of such observations, although the species selected are not necessarily typical of planktonic algae. The quantities produced agree reasonably well with the figure given by Braarud and Føyn (4), who obtained evidence from wet-oxidation studies that, during a 10-week growing period, about 30% of the total organic matter produced by a marine *Chlamydomonas* species was liberated into the medium. Using six fresh-water species of this genus, Allen (1) obtained values which, expressed on a comparable basis, ranged from 10% to 30%. Benecke (2) pointed out that "with every increase of *Chlamydomonas* cells, membranes are cast off, and soluble substances may result from their solution". Disintegrating fragments of cell walls were in fact observed in cultures of *C. moewusii* (17).

The roles of such large organic molecules in nature may be various. Since their production by soil algae has been demonstrated, it is probable that hydrocolloids of this sort contribute to the character of the soil. Their possible action as chelating agents for heavy metals is indicated by the frequent presence of uronic acid residues in the molecule, and is supported by some preliminary observations of Pollard and Smith (22). Similar activity has been demonstrated by Fogg and Westlake (6) for polypeptides. In lakes or seas, such substances may influence phytoplankton succession directly, in the manner indicated by Lefèvre, Jakob, and Nisbet (14), or, by providing substrates for the growth of heterotrophic bacteria, they may produce indirect effects on the subsequent algal flora.

Critical studies of the effects of chemically characterized algal metabolites on other organisms must be made before we can hope to understand some of the underlying causes of phytoplankton succession.

Acknowledgments

The author wishes gratefully to acknowledge cultures of *Chlamydomonas dysosmos* from Mr. I. C. G. Cooper, and of *C. moewusii* from Dr. L. Provasoli; soil samples from the Mexico region collected by Dr. J. Reeder, and from the Arctic shores by Lieut. R. Lindsay of the 1954 Canadian "Labrador" expedition; the identification of *Chlorosarcina* (*Chlorosphaera*) *consociata* by Dr. G. W. Prescott; advice in chromatographic methods from Dr. A. N. O'Neill; laboratory assistance from Miss Rita H. Mintz; and valuable guidance in the writing of this report from Dr. E. G. Young.

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STUDIES ON POLYSACCHARIDE FORMATION BY PSEUDOMONAS FLUORESCENS[1]

By R. G. Eagon

Abstract

Pseudomonas fluorescens, strain OSU 64, was shown to produce only a mannan regardless of the type of carbon source employed as the nutriment. Stationary cultures were more favorable for polysaccharide formation than shaken cultures. Growth curve data indicated that polysaccharide production took place after the phase of logarithmic growth. Methylpentose was demonstrated to be present in the cells of this organism.

Introduction

Eagon and Randles (5) first reported that a strain of *Pseudomonas fluorescens* produced a slime when grown on sodium acetate. This slime was shown to be a polysaccharide which yielded only mannose when hydrolyzed by mineral acids. At that time, it was observed that slime was always formed when *P. fluorescens* was grown on salts of organic acids but not when grown on carbohydrates. Later work (unpublished) indicated that an alkaline reaction (pH 8) and/or a high concentration of potassium or sodium ions were necessary in order for slime to be produced when this organism was grown on sugars. No attempt was made at this time to determine whether the composition of the polysaccharide was influenced by the substrate.

Experiments conducted by Eagon and Dedonder (3, 4) with several other strains of *P. fluorescens* indicated that all strains tested produced polysaccharides and that these polysaccharides differed one from another.

The purpose of the present work, therefore, was to determine if cultural conditions and substrate influence the composition and quantity of polysaccharide formation.

Experimental Methods

The Microorganism and Media Employed

The organism used throughout this investigation was *Pseudomonas fluorescens*, strain OSU 64, which previously had been shown to form a mannose polysaccharide when grown on acetate (5). A basal medium was prepared according to Grelet (6) to which could be added organic substrates.

Harvesting and Purification Procedures of the Polysaccharide

In order to obtain the polysaccharide, the culture medium was first agitated a few seconds in a Waring blendor and then the cells were removed by centrifugation. The polysaccharide was then precipitated with alcohol from this cell-free supernatant.

[1]*Manuscript received July 13, 1956.*
Contribution from the Department of Bacteriology, the University of Georgia, Athens, Georgia, U.S.A.

The polysaccharide could be purified by boiling three to five minutes in 1% sodium hydroxide followed by reprecipitation with alcohol and dialysis against distilled water. Purification could also be achieved by successive alcohol reprecipitations of water solutions. The end products gave negative ninhydrin reactions.

Identification of Mannose

A variety of tests were employed to identify mannose. All results seemed to indicate that the polysaccharide produced by acetate-grown cells was composed solely of mannose subunits. Only the single spot corresponding to mannose was revealed when paper chromatograms were developed with either phenol or butanol–ethanol–water solvents.

Unlike aniline phthalate, silver nitrate spray reagent does not depend upon reducing groups. Additional spots failed to appear when silver nitrate was used, indicating the absence of compounds other than mannose.

The hydrolyzate was ninhydrin negative, indicating the absence of amino sugars. Dische's carbazol reaction for hexuronic acids was negative (1) and, using the unhydrolyzed polysaccharide, Kapp's method for the micro-estimation of uronic acids was also negative (8). In addition, both Tollen's phloroglucinal test for pentoses and Saliwanoff's test for keto-sugars were negative.

Using conventional technique, a white crystalline product was prepared from the hydrolyzate. Phenylhydrazine derivatives were prepared. A precipitate formed from the phenylhydrazine reaction mixture in less than a minute. This was taken to be mannose phenylhydrazone and is a reaction characteristic of mannose, other sugars forming soluble hydrazones. Upon further reaction there was formed an insoluble osazone which gave a melting point of 204°–205° C., which is characteristic of mannose. There was no deviation when a mixed melting point was made.

Although difficult to perform because of the small amount of material available, optical rotation determinations seemed to indicate that the rotation was approximately +15°, which is that of mannose.

Since mannose oxidation by *P. fluorescens* is adaptive, adaptive enzyme studies could be used to further identify mannose. Consequently, cells grown on hydrolyzate were adapted to mannose and oxidized mannose to the same rate and extent as the hydrolyzate. The same was true for the reverse situation, e.g., mannose-grown cells were adapted to the hydrolyzate.

Techniques Used in the Study of Polysaccharides from Other Sources

Since mannose was so clearly indicated, all additional experiments depended upon chromatographic methods solely. The polysaccharide was hydrolyzed in 1% sulphuric acid at 100° C. for three hours. Paper chromatograms were developed with a solvent composed of butanol, ethanol, and water (40 : 11 : 19 by volume) and the spray reagents used were either aniline phthalate or silver nitrate.

Results and Discussion

Identification of "Slimes" Produced from Various Substrates

Slimes were collected from the culture media containing such varied carbon sources as hexoses, pentoses, organic acids, e.g., gluconate, succinate, citrate, etc., and glycerol. Paper chromatography of the hydrolytic products of these polysaccharides yielded only mannose. Furthermore, the quantity of polysaccharide produced appeared to be equal for all substrates, approximately 1.2 gm. per liter of medium, employing stationary culture conditions.

Comparison of Physical Cultural Conditions

An experiment was designed to determine whether stationary or shaken cultures most efficiently produced polysaccharide. To the basal medium was added 6% glucose and 0.4% yeast extract. The stationary cultures consisted of 1 liter Erlenmeyer flasks containing 100 ml. medium. The shaken cultures were 500 ml. Erlenmeyer flasks containing 100 ml. medium; a rotary-type shaker was used. The cultures were incubated for 72 hr. at 30° C. Table I summarizes the results of these experiments. Estimation of glucose was accomplished by means of the anthrone reagent. It is indicated that whereas shaken cultures produced more cells and utilized more glucose than stationary cultures, less polysaccharide was produced. A similar phenomenon has been observed by Haynes for *P. aeruginosa* (7). This author stated that slime was seldom observed in shake flasks and became evident only upon standing.

TABLE I

COMPARISON OF SHAKEN AND STATIONARY CULTURES

	Glucose utilized, gm./liter	Cells produced, gm./liter	Polysaccharide produced, gm./liter
Shaken cultures	15.0	4.0	0.7
Stationary cultures	6.6	2.2	1.2

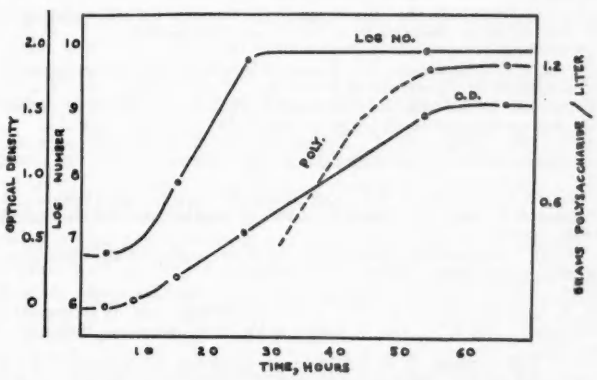

FIG. 1. Growth curve of *P. fluorescens* as related to polysaccharide production.

Growth Curve Data

Earlier observations had indicated that polysaccharide formation did not occur until after maximum growth occurred. The results indicated in Fig. 1 seem to justify this assumption. Stationary cultures were employed at 30° C. It is seen here that the viable cell count leveled off in 24 hr. but that the optical density continued to increase for another 24 hr. Polysaccharide formation must have taken place late in the logarithmic growth phase and during the stationary phase. In this respect, it behaves much like inclusion formation and perhaps could serve as an additional argument for the theory of *metabolic shunts* as an explanation for the origin of inclusions and slime layers (9).

Carbohydrate Impurities Contained in the Crude Polysaccharide

Paper chromatography of the hydrolyzate of unpurified polysaccharide usually revealed two additional spots other than mannose. One spot had an R_f value equal to that of ribose and when sprayed with aniline phthalate yielded a pink color characteristic of pentoses. The second spot was yellowish in color and had a movement similar to that of rhamnose. Both spots disappeared when the polysaccharide was dialyzed against distilled water, indicating that these substances were contaminants, possibly resulting from autolysis of cells.

Since the methylpentoses, fucose and rhamnose, have been shown to be produced by certain strains of *P. fluorescens* (3, 4), the Dische-Shettles method was employed to assay for methyl pentose in this organism (2). Results revealed that these cells contained 1% of methyl pentose by dry weight. This substance might possibly function as a cell wall constituent, as was recently demonstrated in the case of *Streptococcus pyogenes* by Slade and Vetter (10).

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STUDIES ON H. PERTUSSIS LIQUID CULTURES

III. LOCALIZATION OF SURFACE ANTIGENS BY MEANS OF FLUORESCENT ANTIBODY[1]

By J. de Repentigny and A. Frappier

Abstract

The fluorescent antibody technique was used in an attempt to clarify the relationship between the morphology of the surface of *Haemophilus pertussis* and the antigenic properties of its capsular antigens. First, specific antibodies were produced by injection into rabbits of agglutinogenic and protective surface washings of the bacilli. Then, these antibodies were made fluorescent and used to mark specifically and *in situ* the original capsular antigens at the surface of bacilli grown in liquid as well as on solid media. Thus was obtained morphological and specific evidence for the presence of a capsule containing antigenic material.

Introduction

Material present at the surface of *Haemophilus pertussis* has been variously described as a capsule or a slime layer (Lawson (13), Evans and Adams (3), Klieneberger-Nobel (12)). Unfortunately, the isolation of this surface material and the study of its antigenic properties have been undertaken mostly by other workers (3, 16, 18); there is no evidence to show that the surface material is identical with the capsule nor even that the capsule itself or the slime layer, as observed by non-specific staining, is antigenic. A more detailed review of the previous work on this subject will be taken up in the discussion. Studies initiated in our laboratories concerned a systematic investigation of the distribution of toxic and protective antigens in fluid cultures of *H. pertussis*. The supernatant of such cultures, the bacilli themselves, and the washings of the bacilli were all analyzed. Frappier and Guérault (6, 7, 8, 9) were thus able to show that merely washing with water a given quantity of bacilli grown in *liquid medium* yielded as much protective antigen as an extract obtained by Hughes' method (10) from an identical quantity of the washed bacteria. Electron microscope studies revealed that washing left the bacterial wall and cytoplasm unchanged; this morphological control of the bacteria before and after washing was suggestive that this washing process removes mostly surface antigens (with no surface alteration visible under the electron microscope), possibly capsular or slime material similar to that formerly described for bacilli grown on solid medium.

In order to verify whether the washings contain capsular material, whether also this same material is antigenic, and whether growth in a liquid medium or washing with water entirely removes the capsule, we took advantage of the precise, sensitive, and specific fluorescent antibody technique. With the use of this method, we thought it might be possible (1) to bring some degree of

[1]*Manuscript received July 16, 1956.*
Contribution from the Institute of Microbiology and Hygiene of the University of Montreal, Montreal, Quebec. This research was supported in part by a grant from the Ministry of Health of the Province of Quebec (Federal–Provincial Grants for Public Health Research).

precision and find a missing link in this complex and ill-defined problem of the surface antigens of *H. pertussis* (19); (2) to determine the cytological position of the extracted antigens.

Our experimental approach was the following: we first prepared rabbit antisera against the bacterial washings (these antisera contained agglutinins and protected mice against an experimental infection). Subsequently, the gamma-globulins isolated from the above antisera were made fluorescent and used to mark specifically and *in situ* the corresponding antigens on the surface of *H. pertussis*. We thus succeeded in tracing back to the surface of the bacilli the origin of the antigens which were extracted by washing, without visible damage to the cell wall.

Materials and Methods

Antigens

(1) Four-day-old cultures of Phase 1 *H. pertussis* (local strain No. 310) were grown on a modified Hornibrook liquid medium.* The bacilli were collected at room temperature using a Sharples continuous flow centrifuge, at 20,000 r.p.m.

(2) The surface washings were obtained by resuspending and shaking the bacilli in the rotor itself with a volume of twice-distilled water corresponding to 1/25th of the volume of the original culture; this operation was carried out manually and in a gentle way for not more than three minutes. The liquid extract was immediately separated from the bacilli by two successive one hour centrifugations, at 4000 r.p.m. and 20,000 r.p.m. in the cold (+ 4° C.), using the International PR2 centrifuge. This material was generally non-toxic when injected intraperitoneally into mice (7). The washed bacilli were still capable of killing the mouse although to about a 15% lesser degree (8) than could the unwashed bacilli, in spite of the fact that the washing process did not diminish the number of live bacteria. Electron microscope examinations revealed no alteration of the bacterial wall after washing (7, 9).

(3) Another extract was obtained at −40° C. from the above-mentioned washed bacilli, by crushing the cells in the press described by Hughes (10); the material was treated with the same proportional quantity of water and under the same conditions as those described for the washing of intact bacilli. This solution was called the "somatic extract" and was highly toxic for mice (9).

(4) Both the surface and somatic extracts, the latter having been detoxified with formol, protected the mouse. These extracts were the very same as those previously used by Frappier, Guérault, and de Repentigny (9). When allowed to diffuse in agar against a purified anti-*H. pertussis* serum, the washings showed two lines and the somatic extract several more (8). Only the two former antigens could be shown to be present in the washings as well as in the somatic extract from washed cells. There were other more deeply situated antigens which could be shown to be present exclusively in the somatic extract besides the two above-mentioned common antigens.

*The $CaCl_2$ content has been reduced to 0.0005%.

Fluorescent Antibodies

(1) Three rabbit antisera were prepared: the first, against fluid cultures of *H. pertussis* inactivated by heat and formaldehyde; the second, against the surface washings; and the third, against the somatic extract. They all were purified (11) in order to separate the gamma-globulins.

(2) Fluorescein isocyanate II was prepared (1, 2) and coupled (1) with these immune-globulins. Fluorescent normal gamma-globulin of rabbits was also prepared; all these gamma-globulins were purified again by two precipitations with ethanol in the cold (15). As expected, the conjugated *normal* gamma-globulin could not agglutinate *H. pertussis*, but the other conjugated antibodies (described in (1)) gave agglutination titers ranging from 1/320 to 1/640.

Staining Procedure

A loopful or a drop of a suspension of *H. pertussis* was placed on a non-fluorescing slide and dried very gently; a drop of fluorescent antibody was deposited on top of it. The slide was allowed to stand in a Petri dish with moist filter paper for three hours at +4° C.; then the specimen was delicately washed with distilled water and dried again very gently.

Fluorescence Photomicrography

Visual observation and photomicrographs of the fluorescent bacteria were made with the Leitz fluorescence microscope, model BX, complete with the photomicrographic attachments. A dark-field condenser and Euphos cover glasses (as ultraviolet filters) proved to be an adequate setup. Buffered glycerol was used between the slides and the cover glasses and also as the immersion liquid. Exposure on Kodak Plus X, 35 mm. film, was for five minutes, at a magnification of 550 ×; undiluted Kodak Dektol was used as developer at a temperature of 24° C. for eight minutes.

Electron Microscopy

The technique of Mudd, Heinmets, and Anderson (17) was followed. The electron microscope was an RCA EMU2C.

Results

When *H. pertussis* cells grown either in a modified Hornibrook liquid medium or on Bordet-Gengou solid medium were treated with purified fluorescent rabbit antibodies (gamma-globulin) prepared respectively against surface washings, somatic extract, and intact *H. pertussis*, a yellow-green fluorescent layer was observed around the bacteria examined with the help of U.V. rays. Fluorescence photomicrographs 1, 2, and 3 illustrate some of these observations. When the U.V. rays were cut off by a filter inserted between the arc lamp and the mirror of the microscope or when fluorescent rabbit normal gamma-globulin was used for the treatment, the yellow-green fluorescence vanished and could not be photographed under our experimental conditions.

The antibodies separately prepared against the three above-mentioned antigens coated the unwashed bacilli in an identical manner. These results

were likely due either to only one or to both of the above-mentioned common antigens shown by the agar diffusion method to be present in the washings as well as in the intact bacilli and in the somatic extract of washed bacilli (9). In all three cases, fluorescence was limited to the outer layer of the bacilli. No fluorescence was observed clearly inside the cell wall, even when antisomatic extract antibodies were used for treatment. The membrane impermeability might account (as a hypothetical explanation) for this latter fact.

When fluorescent antibodies prepared against somatic extract of *washed* bacilli were fixed upon *unwashed* bacteria, the outer layer still became fluorescent. This unexpected result suggests that the removal of the surface antigens during the washing of bacilli prior to somatic extraction was incomplete or/and that some antigens are also present inside and outside the cell wall.

In order to find out whether the washing process removes the capsular material entirely, the *washed* bacilli were treated with fluorescent antiwashings antibody. They still showed some fluorescence on the surface. Therefore, surface antigens did not seem to be entirely removed by the washing. This could explain also the presence of surface antibodies in the antisomatic serum.

These latter findings were corroborated by the agar diffusion technique: common lines between the washed bacilli, the washings, and the somatic extract could be observed when these antigens were allowed to diffuse against an antiwashings serum (9).

Electron micrographs were in agreement with these latter findings. The morphology of the cell surface did not change when purified rabbit *normal* gamma-globulin was used (Fig. 5). Antiwashings antibodies induced a visible modification of the outer layer of the unwashed bacilli, even if grown in a liquid medium (Fig. 4); in some respects, this modification could be interpreted as a capsule-swelling (Quellung) reaction. However, under similar experimental conditions, this layer, as seen under the electron microscope, seemed to be smaller than the one shown with the fluorescent antibody technique. Finally, surface antigenic material could be detected on bacilli both before and after washing by using the fluorescent antibody technique.

FIGS. 1–3. Fluorescence micrographs.

FIG. 1. At the surface of *H. pertussis*, grown in a modified liquid medium, antigenic material is revealed through the use of fluorescent rabbit antiwashings antibody. ×2200

FIG. 2. Similar results as in FIG. 1 were obtained when *H. pertussis* grown on Bordet–Gengou medium was used. × 2200

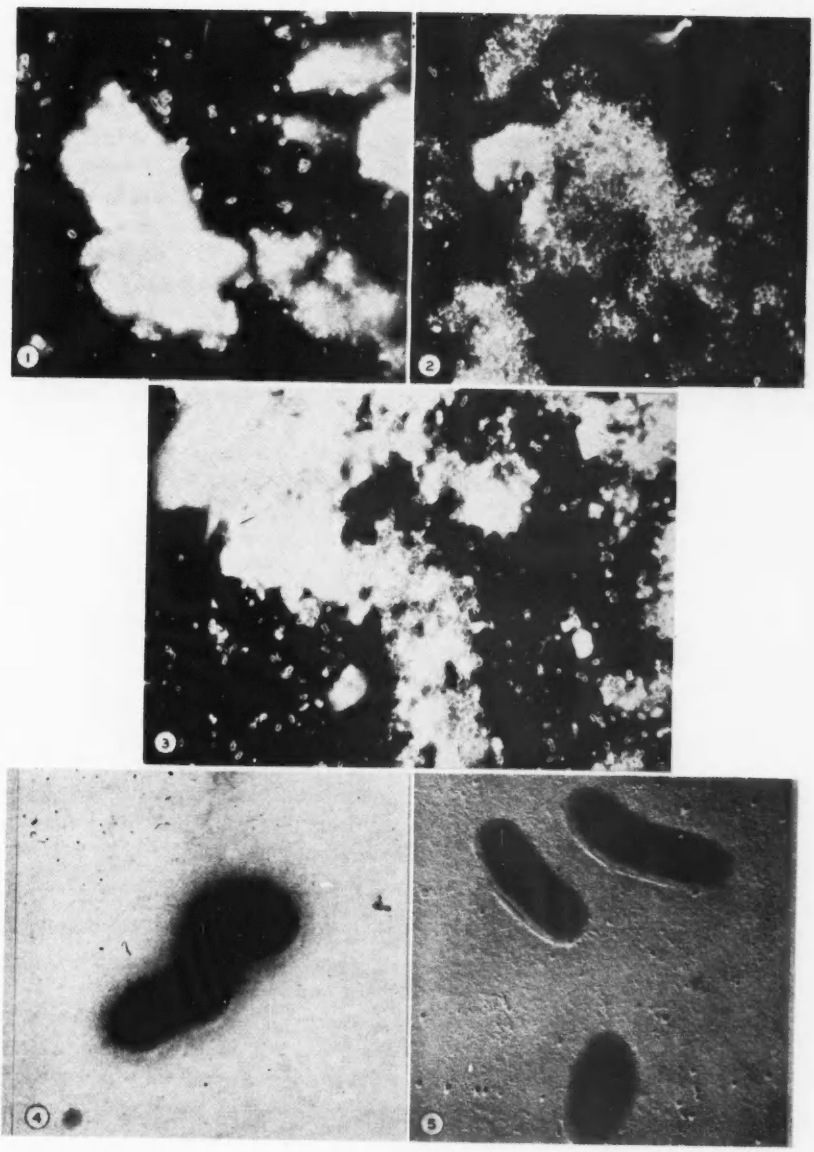
FIG. 3. Fixation of fluorescent antisomatic extract antibody at the surface of *H. pertussis* grown on Bordet–Gengou medium. × 2200

FIGS. 4, 5. Electron micrographs.

FIG. 4. Modification of the outer layer of *H. pertussis*, grown in liquid medium, is observed after treatment with purified rabbit antiwashings antibody. A delicate slime partly distinct from the bacteria can be seen. × 50,000

FIG. 5. There is no visible alteration in the morphology of *H. pertussis*, grown in liquid medium, after it has been treated with purified rabbit normal gamma-globulin. No capsular-like material is apparent. × 50,000

PLATE I



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Discussion and Conclusion

The experimental results herein described bring further and specific evidence of the existence of antigenic material around *H. pertussis*; previously reported work on this subject could only suggest this fact. It seems that no attempt has been made to show if the antiserum prepared against surface material can react specifically and *in situ* with the capsule.

In morphological studies (3, 12, 13), the capsule was demonstrated only by using bacilli grown on solid media and by resorting to an unusual chemical staining technique. Of all the workers who attempted to extract the antigenic capsular materials from such cultures, no one but Evans and Adams (3), to our knowledge, controlled morphologically the existence of the capsule-like material prior to its extraction. The methods of extraction varied with each worker and were in some respects contradictory; therefore surface material so extracted differed widely from one method to another. Lawson (13), Evans and Adams (3), and Miller (16) claimed that the capsular material was removed by a mere washing whereas Smolens and Mudd (18) discarded these very same washings before proceeding to the extraction of the so-called surface material. All these authors submitted the washings or extracts to filtration, a procedure which, according to Miller (16) and Flosdorf and Kimball (5) and our own work (6), retains partially or in whole, different antigens, including the protective antigens; this retention may vary with the kind of filter used or the quantity of antigenic material being filtered.

Under these circumstances, it is likely that the antisera produced against the surface extracts made by these workers differed in their agglutinating and protective properties: for example, antigens isolated by Smolens and Mudd (18) produced highly agglutinating and protective antisera, while those of Evans and Adams (3) showed a low ability to produce agglutinins and did not elicit the production of protective antibodies. In a recent paper, Evans and Perkins (4) seemed to suggest that agglutinogen and protective antigen are present at the surface of the bacilli since they absorb the corresponding antibodies.

Moreover, the above authors themselves stated that chemical staining seemed to be of limited application for demonstrating the presence of a capsule because the addition of water to the bacilli or its use in the staining process washed the capsule away. Bearing this fact in mind, one could not expect, using such a staining technique, to obtain positive evidence of a capsule around the bacilli, if these are grown in a liquid medium.

By using specific fluorescent antibodies, we could obtain positive results with bacilli grown in liquid as well as on solid media. The failure of other authors to stain chemically the capsular material in the presence of water seems due only to the method used.

Maitland, Kohn, and Macdonald (14) have shown that the histamine-sensitizing factor (HSF) of *H. pertussis* could be found in washed as well as in intact bacteria; and, from this, they deduced that this factor was not capsular material. They did not mention whether HSF has been sought

directly in the washings; and if we recall that antigenic material was only partially extracted by our own washing, it may be surmised that the same antigens can therefore be present in the washed bacteria, in the intact bacteria, and in the washings.

To sum up, the use of the specific fluorescent antibody technique, in conjunction with other methods, has enabled us to obtain the following results concerning the antigenic structure of *H. pertussis*: (*a*) additional experimental evidence that *H. pertussis* is surrounded by a capsule when grown on solid medium; and, for the first time, evidence that the capsule is present even when bacilli are grown in liquid media; (*b*) specific evidence that this capsule contains antigenic material which can be extracted by a mere washing with water; (*c*) such washing does not, however, seem to remove entirely the antigenic capsular material; agar diffusion tests revealed that common antigens are present in the capsule of the unwashed bacteria, and inside the cell wall of the washed bacteria; (*d*) the antigenic material we have found in the surface washings shows protective and agglutinogenic properties; (*e*) the specific fluorescent antibody technique is a method capable of giving clear and specific demonstration of the presence of antigenic capsular material when chemical staining may fail and electron microscopy might give less striking evidence.

Acknowledgments

The authors wish to express their thanks to Dr. A. Guérault and Dr. S. Sonea for their helpful suggestions and for reviewing the manuscript.

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STUDIES ON MYCOBACTERIA

I. CYTOCHEMICAL STUDY AND CHEMICAL ANALYSIS OF THE LIPIDS OF MYCOBACTERIUM MARIANUM[1]

By Soeur Marie Suzanne, V. Portelance, and M. Panisset

Abstract

Several authors have reported that the composition of the lipids of the tubercle bacillus varies with the type and composition of the culture media. Major differences may therefore exist between the bacillary lipids of bacilli growing on culture media and those of such microorganisms parasitizing a host.

The present paper deals with the cytochemical and chemical analyses of *Mycobacterium marianum*, which is, to our knowledge, the first strain of *Mycobacterium* to be stained by the Sudan black B method when grown on Sauton's medium. Details on the isolation and fractionation of several lipid fractions of *Mycobacterium marianum* are presented together with some of their properties. The results are discussed in relation to our working hypothesis that the chemical composition of *Mycobacterium marianum* grown under our conditions may resemble more closely that of bacilli growing *in vivo* than do most cultured mycobacteria.

Introduction

The chemical composition of the tubercle bacillus has been very extensively studied in the last decades and the work of Anderson (3, 4, 5) and his school predominates in the field of bacterial lipids. His results, together with those of Chargaff and Dieryck (10), Frouin and Guillaumie (13), Stephenson and Whetham (23), have shown, without any doubt, that there exists an extremely close relationship between the composition of the culture medium and that of the bacillary lipids. Consequently, one may postulate that the composition of a bacillus growing in a living tissue is quite different from that of an organism cultured in the laboratory. Such an hypothesis is supported by cytochemical reactions among which is the Sudan black B staining technique reported by Sheehan and Whitwell (22). These authors observed that *Mycobacterium tuberculosis* from lesions or in sputum is stained by Sudan black B while such organisms remain unstained by the same method when they are harvested from laboratory cultures.

We were deeply interested by this observation, and in the last two years we have tried to modify Sauton's culture medium to obtain mycobacteria that would be stained by Sudan black B (21); but we have thus far been unsuccessful. Evidently, bacilli isolated from tuberculous lesions would be the ideal material for chemical analysis, but the large numbers of bacilli necessary for this study is unavailable in such lesions, a fact that might explain the failure of Anderson, Reeves, Creighton, and Lothrop (6), who tried to isolate from pulmonary lesions some constituents characteristic of the tubercle bacillus grown on culture media (tuberculostearic and phtioic acids, phtiocol).

[1]*Manuscript received June 5, 1956.*

Contribution from Laboratoire de recherches sur la lèpre, Facultés catholiques de Lyon, 25, rue du Plat, Lyon, France (Soeur Marie Suzanne) and The Institute of Microbiology and Hygiene, University of Montreal (V. Portelance and M. Panisset). This research was partially aided by grant from the Ministry of Health of the Province of Quebec (Federal Provincial Health Research Grants).

A few years ago, one of us (S.M.S.) (17) isolated a strain of *Mycobacterium* from lepromas. This strain, which was called *Mycobacterium marianum*, can be grown easily on culture media, is very strongly and evenly acid-fast, and is stained by the Sudan black B method when grown on Sauton's medium. This is, to our knowledge, the first strain of *Mycobacterium* which, even when grown under these experimental conditions, can be stained by Sudan black B. The study of *Mycobacterium marianum* was thus undertaken to discover whether any unique chemical characteristics could be detected.

The preliminary results of the cytochemical and chemical analyses of *Mycobacterium marianum* have already been reported (20) and the present paper gives the results of our first full series studying the lipids of *Mycobacterium marianum*.

Materials and Methods

(*A*) *Culture and Harvesting of the Bacteria*

Five hundred 250 ml. culture flasks each containing 150 ml. of Sauton's medium were seeded with *Mycobacterium marianum* and incubated at 37.5° C. Two hundred and fifty flasks of the culture were harvested after 28 days of growth; the remaining 250 flasks were left for a forthcoming study.

The 250 flasks studied were divided into four lots which were treated separately. The bacterial mass, which consisted of a heavily pigmented (golden-yellow) and slimy culture, had to be harvested by centrifugation at 3000 r.p.m. for 20 min. The supernatant was decanted, and the bacterial sediment was washed twice with distilled water in order to remove any trace of medium. The recentrifuged live bacilli were finally transferred to a Buchner funnel and dried in air for two hours. The combined supernatants and washings were filtered through a Seitz sterilizing pad and stored at +5° C. for a study of the protein content, the results of which will be published later.

(*B*) *Cytochemical Study*

A loopful of the bacterial growth was emulsified in one drop of Sauton's medium on a glass slide and dried in air. The smears were divided into two series, one of which was treated with various organic solvents for 24 hr. at room temperature. Both the treated and untreated smears were stained with Sudan black B following the technique devised by Sheehan and Whitwell (22).

The lipid fractions of *Mycobacterium marianum* were emulsified in one drop of chloroform on a glass slide, dried in air, and stained by Sudan black B following the modified method of Chiffelle and Putt (12). Controls were made with smears of *Mycobacterium marianum*.

(*C*) *Chemical Study*

(*a*) *Extraction and Fractionation of the Lipids*

The live air-dried bacilli were extracted and fractionated by the method of Anderson as modified by Aebi, Asselineau, and Lederer (1). The technique may be summarized as follows:

The air-dried bacilli were placed in a mixture of ethanol–ether (1 : 1) using 25 ml. of solvent per culture flask harvested, the bacillary mass of which weighed approximately 0.6 gm. (dry weight). After complete extraction by this solvent, the extracts were concentrated *in vacuo*, dissolved in ether, and the ether solution washed once with water. The ether-soluble portion was separated into three fractions:

1. Fat, soluble in cold acetone.
2. The A waxes, soluble in hot acetone.
3. Phosphatides, insoluble in hot acetone.

It should be well understood that the specific chemical terms "fat", "phosphatide", and "wax" are knowingly used loosely, each of them having been ascribed for the first time to extremely complex crude fractions by Anderson, based on the physical aspect of the fractions and their solubility in organic solvents. These terms have thereafter been used by all authors working on the lipids of mycobacteria even though it is well known that these fractions are far from being pure.

The bacillary residues left after extraction with ethanol–ether were thereafter repeatedly extracted with chloroform. The chloroform extracts were evaporated to dryness *in vacuo* and the residue was separated into three fractions:

1. The B waxes, soluble in a mixture of ether–methanol.
2. The C waxes, soluble in hot acetone.
3. The D waxes, insoluble in hot acetone.

(*b*) *Analytical Techniques*

1. The colorless lipid fractions were fractionated by the "liquid chromatogram" technique of Reichstein (19). The alumina used as adsorbent was prepared by the technique of Asselineau (7) and standardized by the method of Brockmann (9). The series of solvents used were as described by Asselineau (7).

The phosphatides were purified by filtration through cellulose powder (14) and chromatographed on silicic acid – celite (16) essentially as described by Michel and Lederer (18).

2. The melting points were determined under the microscope with the Köfler apparatus and are hereafter designated M.P.

3. Acidity of the fractions was determined with a Lindström-Lang type of ultra-microburette; the technique was as described by Asselineau (7) and the acidity is expressed in cubic millimeters of 0.1 *N* KOH per milligram of product.

4. Tentative molecular weights were determined by titration with 0.01 *N* sodium hydroxide and are hereafter designated T.M.W.

5. The phosphorus content was determined by the method of Allen (2).

Results

(A) Cytochemical Study

The results of the cytochemical study made on treated smears of *Mycobacterium marianum* by various organic solvents for 24 hr. at room temperature are summarized in Table I.

The results of staining by the Ziehl-Neelsen method agree with those obtained by others who have studied the acid-fastness of mycobacteria. While studying the stainability by Sudan black B, it was interesting to refer to the paper of Sheehan and Whitwell (22). These authors observed that tubercle bacilli in tissues and sputa retained the Sudan black B even after treatment for 24 hr. with acetone, but that this property was lost when the smears were treated by various other solvents. Our results with *Mycobacterium marianum* were identical. This suggests that one or more acetone-insoluble substances with unique staining characteristics are involved.

TABLE I

STAINABILITY OF *Mycobacterium marianum* AFTER TREATMENT WITH ORGANIC SOLVENTS FOR 24 HR.

Solvents	Ziehl-Neelsen	Sudan black B
Ethanol	+	±
Ethanol (HCl 3%)	0	±
Methanol	+	±
Propylene glycol	+	±
Butanol	+	±
Diethylene glycol	+	±
Diacetin	+	±
Dioxane	+	±
Xylene	+	±
Chloroform	+	±
Lutidin	+	±
Methylcellosolve	+	±
Ether	+	±
Acetone	+	+
Bayol F	+	±
Untreated smears of *M. marianum*	+	+
Untreated smears of BCG (from Sauton's medium)	+	±
Untreated smears of tubercle bacilli from sputum	+	+

NOTE: + *means normal acid fastness or sudanophilia.*
± *means gray staining by Sudan black B.*
0 *means total loss of acid fastness or sudanophilia.*

(B) Chemical Study

(*1*) *Extraction of the Lipid Fractions*

Following the scheme of extraction previously outlined, each lot of bacilli was treated separately and Table II gives a summary of the concentration of several lipid fractions isolated from *Mycobacterium marianum.*

TABLE II

LIPID FRACTIONS OF *Mycobacterium marianum* (28 DAYS ON SAUTON'S MEDIUM) EXPRESSED AS % OF DRY WEIGHT OF BACILLI

Lipid fractions	Lot I	Lot II	Lot III	Lot IV	Average
Ethanol–ether extract	13.00	13.55	12.68	13.29	13.13
1. Acetone-soluble fat	7.18	8.23	7.79	8.01	7.80
2. The A waxes	1.87	1.58	0.99	1.48	1.48
3. Phosphatides	3.95	3.73	3.89	3.78	3.84
Chloroform extract	2.28	2.69	2.20	1.77	2.27
Total free lipids	15.29	16.25	14.88	15.06	15.37

TABLE III

LIPID FRACTIONS OF MYCOBACTERIA GROWN ON SAUTON'S MEDIUM FOR 28 DAYS (AS % OF DRY WEIGHT OF BACILLI)

	Strains and virulence						
	*M. marianum**	Brévannes* +++	H37Rv* +++	H37Ra* −	BCG† −	Vallée† +	Marmorek† +
	The authors	Asselineau				Demarteau	
Ethanol–ether extract	13.13	14.1	12.7	9.6	11.0	6.0	5.6
1. Acetone-soluble fat	7.80	6.6	—	5.6	8.8	3.3	3.4
2. The A waxes	1.48	5.3	—	2.2	1.7	1.2	1.6
3. Phosphatides	3.84	2.2	—	1.8	0.5	1.5	0.6
Chloroform extract	2.27	9.65	8.4	6.5	9.0	8.3	10.5
Total free lipids	15.37	23.8	21.1	16.1	20.4	14.3	16.1

* = *Human strain.*
† = *Bovine strain.*

Table III gives a comparison of these results with those of other authors as cited in the literature and obtained with several strains of mycobacteria of human and bovine origin and of varying virulence.

It can be seen that the total free lipid content of *Mycobacterium marianum*, cultured on Sauton's medium for 28 days, is 15.3%, a content which is similar to that of other mycobacteria. Hence *Mycobacterium marianum* seems to be more closely related to the non-virulent, or low-virulence, strains of mycobacteria, whether of human or bovine origin, than to the strains of high virulence.

As far as the acetone-insoluble fractions are concerned, we see that the percentage of phosphatides of *Mycobacterium marianum* is somewhat higher than that of other mycobacteria as reported in Table III, but the content of 3.8% found for *Mycobacterium marianum* is of the same magnitude as the percentage of phosphatides found in our laboratories for the BCG, H37Rv,

and H37Ra strains. On the other hand, *Mycobacterium marianum* contains less of the D waxes, which normally constitute the major portion of the chloroform extract (15). On these grounds, the phosphatides and the D waxes seemed interesting for a detailed analysis, and the desirability of such a study was further indicated by the previous finding (20) that the phosphatides of *Mycobacterium marianum* strongly retained Sudan black B while those from the BCG strain remained unstained and those from the H37Rv strain were very slightly stained. This information seemed to indicate a possible qualitative difference between the phosphatides of *Mycobacterium marianum* and those of other mycobacteria studied from culture media.

(2) *Study of the Lipid Fractions*

To study further the fractions already isolated, corresponding fractions of each lot were pooled. The following study was made therefore on acetone-soluble fats, A waxes, phosphatides, and chloroform extracts pooled respectively.

(a) *The Alcohol–Ether Extract*

1. *The acetone-soluble fats* consisted of a very heavily pigmented (deep brown) material, the acidity of which was 3.5 mm.^{3} KOH 0.1 *N* per mgm. of product. Acetone-soluble fats (10.99 gm.) were mixed with 0.9 gm. of a residue obtained from the purification of the A waxes, a residue which resembled the acetone-soluble fats. The mixture (11.9 gm.) was dissolved in 500 ml. of ether and the solution was washed:

(i) Ten times with 50 ml. portions of a 10% sodium bicarbonate solution. The aqueous extracts were washed with ether and the ether layer was separated from the aqueous one. The alkaline solutions were only pale yellow, thus indicating the absence of free phthiocol, which, if present, would have given a pink solution. The alkaline solution was acidified with sulphuric acid (diluted $\frac{1}{3}$) and extracted with ether. The ether solution was dried over anhydrous sodium sulphate and the ether distilled off, giving 0.1233 gm. of residue (1% of the fat).

(ii) Twice with 150 ml. portions of a 5% sodium carbonate solution. After the usual treatment, 0.062 gm. of residue was obtained (0.5% of the fat).

(iii) Nine times with a 2% potassium hydroxide solution (in 110 ml. portions). The usual treatment gave 2.4836 gm. of fatty acids, which represented 20.87% of the acetone-soluble fats.

(iv) The residual ether solution was washed with water until neutral, dried, and the ether distilled off leaving 9.05 gm. of neutral fats (76% of the fats).

Total recovery of the fat: 98.5%.

CHROMATOGRAPHY I

Neutral fats: 9.0128 gm. dissolved in 100 ml. of petroleum ether.
Neutralized alumina (Activity II): 270 gm.
Elution of 500 ml. of solvent.

	Solvents	Weight of the fractions, gm.	Remarks
1.	Petroleum ether	5.9032	Pale yellow solid
2.	Petroleum ether	0.0949	" " "
3.	Petroleum ether – benzene (9 : 1)	0.1741	" " "
4.	" " " (4 : 1)	0.1857	" " "
5.	" " " (1 : 1)	0.2413	Orange solid
6.	Benzene	0.2705	Red solid
7.	Benzene – ether (9 : 1)	0.2915	Yellow solid
8.	" (1 : 1)	0.5060	" "
9.	" (1 : 1)	0.1120	" "
10.	Ether	0.0410	" "
11.	"	0.0071	" "
12.	"	0.0104	" "
13.	"	0.0066	" "
14.	Ether – acetic acid 1%	0.4478	Brown solid
15.	" " 2%	0.2609	" "
16.	" " 10%	0.2108	" "
17.	" " 10%	0.0858	" "
18.	" " 10%	0.0728	" "
	Recovery	8.9224 (98.9%)	

These fractions were combined as follows to prepare for a more elaborate chromatography.

(*a*) Solvent: petroleum ether; fractions 1 and 2.

(*b*) Solvent: petroleum ether – benzene; fractions 3, 4, 5.

(*c*) Solvent: benzene, benzene–ether, ether; fractions 6 to 13.

(*d*) Solvent: ether – acetic acid; fractions 14 to 18.

The chemical analysis of these fractions is now in progress.

2. *The A waxes* of *Mycobacterium marianum*, a white solid of low acidity (1.0 mm.^{3} 0.1 *N* KOH per mgm.) and of M.P.: 42.5–46° C., were chromatographed and saponified.

CHROMATOGRAPHY II

Weight of A waxes: 0.9377 gm. (dissolved in petroleum ether).
Neutralized alumina (Activity II): 19 gm.
Elutions of 100 ml. of solvent.

	Solvents	Weight of fractions, gm.
1.	Petroleum ether	0.1723
2.	" "	0.1933
3.	" " – benzene 30%	0.0768
4.	Benzene	0.0725
5.	Ether	0.0552
6.	"	0.0114
7.	Ether – methanol 20%	0.2774
8.	" " 20%	0.0287
9.	" " 50%	0.0050
10.	Ether – acetic acid 5%	0.0332
	Recovery	0.9258 (98.7%)

The combined fractions 1 and 2 and fraction 7 were dissolved in ether and precipitated by methanol.

Fractions 1 + 2: white solid, M.P. 44°–48° C., neutral.

Fraction 7: white solid, M.P. 37°–44° C., acidity: 0.155 mm^3. 0.1 *N* KOH per mgm.

For saponification of the A waxes 0.9846 gm. of unchromatographed A waxes was dissolved in 10 ml. of benzene, and 10 ml. of a 5% methanolic potassium hydroxide solution was added. The mixture was refluxed on a water bath for 20 hr., poured into 50 ml. of water, acidified with hydrochloric acid, and extracted with ether (four portions of 100 ml.). The ether soluble portion (0.9846 gm. or 100%) was redissolved in 25 ml. of ether and precipitated by 2 volumes of methanol. After the precipitation had been completed in the refrigerator, the precipitate was filtered, washed with cold MeOH, and called "Acid M": weight, 0.5457 gm. (55.42% of the A waxes); M.P., 58.5°–61° C.; acidity, 15.57 mm.^{3} of KOH 0.1 *N* per mgm.; T.M.W., 792. The combined filtrates were evaporated to dryness, dissolved in ether, and the ether solution washed with a 3% potassium hydroxide solution. The ether layer was dried and evaporated to dryness, giving 0.0455 gm. (4.61%) of neutral fraction. The alkaline layer was acidified and extracted with ether. After evaporation, 0.2234 gm. (22.7%) of acid fraction was obtained. The M.P. of this was 37.5°–43° C., and the T.M.W. was 312. Both the "Acid M" and the neutral fraction were chromatographed.

CHROMATOGRAPHY III

Weight of "Acid M": 404.9 mgm.
Neutralized alumina (Activity II): 10 gm.
The product being only slightly soluble in petroleum ether, carbon tetrachloride, and benzene, it was dissolved in 40 ml. of ether to be chromatographed.
Elutions of 75 ml.

	Solvents	Weight of fractions, mgm.
1.	Ether	14.4
2.	"	2.4
3.	"	2.5
4.	Ether – acetic acid 10%	283.9
5.	" " " 10%	17.2
6.	" " " 10%	15.6
7.	" " " 10%	41.9
8.	" " " 10%	16.1
	Recovery	394.0 (97.3%)

Fraction 4 is a white solid, M.P.: 61.5°–65° C. Fraction 4 and the combined fractions 5, 6, 7, and 8 were rechromatographed.

CHROMATOGRAPHY IV

Weight of fraction 4 of chromatography III: 283.9 mgm.
Neutralized alumina (Activity II): 10 gm.
The product was dissolved in 10 ml. benzene and elutions of 30 ml. were made.

	Solvents	Weight of fractions, mgm.
1.	Benzene	11.1
2.	Ether	1.0
3.	Ether – acetic acid 0.1%	44.2
4.	" " " 0.1%	27.6
5.	" " " 0.2%	17.6
6.	" " " 0.2%	12.0
7.	" " " 0.3%	12.9
8.	" " " 0.3%	9.6
9.	" " " 0.5%	9.7
10.	" " " 0.5%	11.3
11.	" " " 1%	21.6
12.	" " " 2%	45.8
13.	" " " 2%	36.2
	Recovery	260.6 (91.8%)

The above fractions were combined on the basis of the solvent used for their elution, that is:

Fractions 3 + 4 : designated I ac M,
Fractions 5 + 6 : designated II ac M,
Fractions 7 + 8 : designated III ac M,
Fractions 9 + 10 : designated IV ac M,
Fraction 11 : designated V ac M,
Fractions 12 + 13 : designated VI ac M.

The combined fractions 5, 6, 7, and 8 from chromatography III were rechromatographed by the same method as that used for fraction 4 and the major fraction, eluted with ether – acetic acid 0.1%, was designated VII ac M. All those fractions (I ac M to VII ac M) were dissolved in ether and precipitated with methanol. All precipitates were white solids, the properties of which were:

Fractions	M.P., °C.	T.M.W.
I ac M	52°–57°	978
II ac M	54°–56.5°	1173
III ac M	55.5°–57°	1319.5
IV ac M	57°–62.5°	919
V ac M	64°–66.5°	826
VI ac M	62°–66°	1107
VII ac M	54.5°–57.5°	899

CHROMATOGRAPHY V

Weight of neutral fraction from the saponification of the A waxes: 45.5 mgm.
Neutralized alumina (Activity II): 2.68 gm.
The product was dissolved in 10 ml. of petroleum ether and elutions of 20 ml. were made.

Solvents	Weight of the fractions, mgm.
1. Petroleum ether	35.2
2. " " – benzene 15%	6.3
3. " " " 30%	1.4
4. Benzene	0
5. " – ether 10%	0.4
6. Ether	1.2
7. "	0
Recovery	44.5 (97.8%)

The combined fractions 1 and 2 were crystallized from ethyl acetate giving a first harvest (8.6 mgm.) of fine white needles; M.P.: 56.5°–59.5° C. By concentration of the mother liquors, and recrystallization, 21.7 mgm. of a white solid, M.P.: 38°–41° C., were obtained.

3. *The combined phosphatides* (4.9463 gm.) were treated with 800 ml. of anhydrous ether. The opalescent solution obtained was centrifuged at +5° C. at 3000 r.p.m. for 20 min. giving a white deposit which was separated from the pale-yellow supernatant. The white solid (1.6528 gm.), which represented 33.4% of the phosphatides, had a phosphorus content of 2.59%. The supernatant was concentrated to dryness *in vacuo*. The method of fractionation described by Michel and Lederer (18) was applied to both fractions, which had previously been purified by filtration through cellulose powder columns as follows:

CHROMATOGRAPHY VI

Weight of the less soluble white solid: 1.6238 gm.
Adsorbent: 15 gm. of cellulose powder "Whatman, coarse, ashless".
The product was dissolved in chloroform to make a 10% solution.
Elutions of 25 ml. of chloroform were made.

Fractions	Weight of the fractions, gm.	
1	0.0071	
2	0.7137	
3	0.8234	
4	0.0405	
5	0.0325	
Recovery	1.6172	(99.5%)

CHROMATOGRAPHY VII

Weight of the dry residue: 3.1531 gm.
Adsorbent: 30 gm. of cellulose powder "Whatman, coarse, ashless".
The product was dissolved in chloroform to make a 10% solution.
Elutions of 25 ml. of chloroform were made.

Fractions	Weight of the fractions, gm.	
1	0.0081	
2	0.0033	
3	0.0008	
4	0.9141	
5	1.2946	
6	0.6740	
7	0.1821	
8	0.0291	
9	0.0072	
10	0.0033	
11	0.0027	
Recovery	3.1193	(98.9%)

The combined fractions 2 and 3 of chromatography VI and the combined fractions 4, 5, 6, 7, and 8 of chromatography VII were rechromatographed on silicic acid – celite mixture, the latter reagents being first treated as described by McKibben and Taylor (16).

CHROMATOGRAPHY VIII

Weight of the combined fractions 2 and 3 from chromatography VI: 1.5371 gm.
Adsorbent: silicic acid – celite (2 : 1): 24 gm.
The product was dissolved in 15 ml. of chloroform and eluted in 20 ml. portions.

	Solvents	Weight of the fractions, gm.	
1.	Chloroform	0.0097	
2.	"	0.0228	
3.	Chloroform–methanol 10%	0.0218	
4.	" " 30%	0.2964	
5.	" " 40%	0.9093	
6.	" " 50%	0.0807	
7.	" " 50%	0.0208	
8.	Chloroform–methanol (1 : 1) + 20% ether	0.0721	
9.	" " + 20% ether	0.0446	
10.	" " + 20% ether	0.0416	
	Recovery	1.5198	(98.8%)

These fractions were combined into three groups called fractions A, B, C.

Fraction A: fractions 1, 2, and 3 : M.P.: 54°–57.5° C.
Fraction B: fractions 4, 5, 6, and 7 : M.P.: 154.5°–163° C.
Fraction C: fractions 8, 9, and 10 : M.P.: 179°–182° C.

The melting points here indicated were taken on the dried residues after evaporation of the solvent. Details on the properties of these fractions are summarized in Table IV.

CHROMATOGRAPHY IX

Weight of the combined fractions 4, 5, 6, 7, and 8 of chromatography VII: 3.0939 gm.
Adsorbent: silicic acid – celite (2 : 1): 48 gm.
The product was dissolved in 50 ml. of chloroform and eluted in 50 ml. portions.

	Solvents	Weight of the fractions, gm.
1.	Chloroform	0.0012
2.	"	0.1316
3.	"	0.4618
4.	Chloroform–methanol 10%	0.0065
5.	" " 30%	0.4326
6.	" " 40%	1.1878
7.	" " 50%	0.1339
8.	" " 50%	0.1882
9.	Chloroform–methanol (1 : 1) + 5% ether	0.1418
10.	" " + 10% ether	0.0714
11.	" " + 20% ether	0.0399
12.	" " + 20% ether	0.0262
13.	" " + 20% ether	0.0797
14.	" " + 30% ether	0.0600
	Recovery	2.9626 (95.7%)

These fractions were combined into six groups called fractions D, E, F, G, H, I.

Fraction D: fractions 2, 3, and 4 : Semisolid.
Fraction E: fraction 5 : M.P.: 108°–120° C.
Fraction F: fraction 6 : M.P.: 169°–171° C.
Fraction G: fractions 7 and 8 : M.P.: 99°–140° C.
Fraction H: fractions 9, 10, 11, and 12: M.P.: 79°– 95° C.
Fraction I: fractions 13 and 14 : M.P.: 125°–138° C.

The melting points here indicated were taken on the dried residues after evaporation of the solvent. Details on the properties of these fractions are summarized in Table IV.

Fractions A to I were dissolved in ether and all were completely soluble except fractions B and H, which were only partly soluble and could thus be fractionated further on the basis of solubility differences. Fraction B gave a less soluble fraction, B_1, and a more soluble one, B_2. Fraction H gave a less soluble fraction, H_1, and a more soluble one, which when precipitated from its ether solution with methanol gave a product that could be fractionated in a more soluble portion, H_2, and a less soluble one, H_3.

All the fractions soluble in ether were precipitated from their ether solution with methanol or acetone.

TABLE IV

PROPERTIES OF FRACTIONS A TO I OBTAINED FROM THE PHOSPHATIDES OF *Mycobacterium marianum*

	Less soluble in ether				More soluble in ether							
	A	B		C	D	E	F	G	H			I
Weight of the fractions (gm.)	0.0543	1.3072		0.1583	0.6011	0.4326	1.1878	0.3221	0.2793			0.1397
% of the phosphatides (total wt. 4.7365 gm.)	1.1	27.6		3.3	12.6	9.1	25.0	6.8	5.8			2.9
M.P. before precipitation	54°–57.5° C.	154.5°–163° C.		179°–182° C.	Semisolid	108°–120° C.	169°–171° C.	99°–140° C.	79°–95° C.			125°–138° C.
Treatment with ether: Sub-fractions	Soluble	Less sol. B_1	More sol. B_2	Slightly soluble	Soluble	Soluble	Soluble	Soluble	Less sol. H_1	More sol. $H_2 + H_3$		Slightly soluble
Precipitated from ether solution by:	MeOH		MeOH		MeOH	MeOH	Acetone	Acetone		MeOH Precipitate treated by ether		
										More sol. H_2	Less sol. H_3	
M.P. of the precipitated fractions	40°–45° C.	165°–173° C.	158°–163° C.	170°–171.5° C.	Semisolid	109°–120° C.	175°–177° C.	168°–178° C.	113°–118° C.	105°–120° C.	150°–165° C.	113°–120° C.
% of phosphorus	0.05	3.42	3.14	3.54	0.78	2.76	1.31	3.57	3.36	4.02	4.17	1.61
% of phosphatides	1.1	1.8	25.8	3.3	12.6	9.1	25.0	6.8	2.4	0.5	0.2	2.9
Physical aspect	Brownish fat	White crystals	White powder	White crystals	Brownish fat	Pale-yellow wax	Pale-brown wax	Yellow fat	Yellow crystals	Yellow crystals	White powder	Light-brown crystals
Staining by Sudan black B	+	0	+	0	+++	+++	+	0	0	+	0	+

(*b*) *The Chloroform Extract*

The combined chloroform extracts (3.1655 gm.) were dissolved in a large excess of ether (800 ml.) and the solution centrifuged at +5° C. at 3000 r.p.m. for 20 min. to eliminate the major portion of the bacilli contained in the extracts. The supernatant was concentrated to 40 ml. and precipitated by 2 volumes of methanol and the precipitation completed in the refrigerator. The precipitate was washed with methanol, redissolved in ether, and reprecipitated by 2 volumes of methanol. The precipitate thus obtained was a mixture of the C and D waxes, which were separated by treatment with boiling acetone until the C waxes were completely removed. Cooling of the hot acetone filtrate gave the C waxes; the insoluble residue was the D waxes. The combined ether–methanol filtrates gave, after evaporation, the B waxes. The following fractions were thus obtained:

The B waxes—soluble in ether–methanol;
The C waxes—insoluble in ether–methanol, soluble in hot acetone but insoluble in cold acetone;
The D waxes—insoluble in boiling acetone.

The acetone filtrates obtained after precipitation and filtration of the C waxes gave a very soluble fraction, even in cold acetone, which was called "Residue of the C waxes". The properties of these fractions are summarized in Table V.

The residue of the C waxes was dissolved in ether and precipitated by methanol giving 0.1721 gm. of a neutral white solid; M.P.: 37.5°–43° C. The ether–methanol filtrate left a yellow oil after evaporation. The neutral white solid was chromatographed.

CHROMATOGRAPHY X

Weight of neutral solid from the residue of the C waxes: 143 mgm.
Neutralized alumina (Activity II): 3.8 gm.
The product was dissolved in 10 ml. of petroleum ether and elutions of 20 ml. were made.

Solvents	Weight of the fractions, mgm.	
1. Petroleum ether	75.6	(acidity: 0.6 mm.³ KOH 0.1 *N*/mgm.)
2. " "	4.8	
3. Petroleum ether – benzene 30%	10.8	
4. Benzene	6.1	
5. Benzene – ether 30%	3.5	
6. Ether	2.5	
7. Ether – acetic acid 5%	34.7	(acidity: 1.3 mm.³ KOH 0.1 *N*/mgm.)
8. " " " 5%	4.6	
Recovery	142.6	(99.7%)

Fractions 1 and 7 were dissolved in ether and precipitated with methanol giving:

Fraction 1: white solid, M.P. 44°–48° C.
Fraction 7: white solid, M.P. 45°–47° C.

A more complete chemical analysis of all the lipid fractions mentioned in the section "Results" will be reported in a further paper.

TABLE V

PROPERTIES OF THE FRACTIONS OF THE CHLOROFORM EXTRACT OF *Mycobacterium marianum*

Fractions	Weight, gm.	% of the chloroform extract	% of dry bacilli	M.P.	Remarks
B waxes	0.4589	14.4	0.32	Semisolid	
C waxes	1.1663	36.8	0.81	46°–52° C.	Acidity: 0.7 mm.³ KOH 0.1 *N* per mgm.
D waxes	0.4486	14.1	0.31	38°–39.5° C.	% P: 0.66%
Residue of the C waxes	0.9617	30.3	0.67		

Discussion

The unique cytochemical properties of *Mycobacterium marianum* led us to believe that the chemical composition of this mycobacterium may resemble more closely that of such organisms grown *in vivo* than do most cultured mycobacteria. In our experiments, *Mycobacterium marianum* was heavily stained by Sudan black B and the sudanophilic lipids were altered by all the organic solvents used except acetone when smears of the bacteria were treated at room temperature for 24 hr. These results agree with those of Sheehan and Whitwell (22). Nevertheless, it must be mentioned that the staining by Sudan black B has never been completely clear-cut. Bacilli isolated from lesions were not totally stainable by Sudan black B nor were those from culture media totally unstainable. The difference is a relative one. Such an observation has recently been reported by Chaussinand and Viette (11), who also found that culture media such as those of Lowenstein and Dubos do give cultures of certain known mycobacteria with varying proportions of bacilli stainable by Sudan black B. It is our opinion that those bacilli which are stained are in a special physiological state. Under our experimental conditions, this special state is predominant in *Mycobacterium marianum* since more than 95% of the bacilli of that strain were always stained.

The fact that the stainability of *Mycobacterium marianum* was unaltered after treatment with acetone suggested that the acetone-insoluble fractions might take part in this phenomenon. To verify this possibility, a chemical analysis of the bacteria was undertaken. The free lipid content of *Mycobacterium marianum* cultured for 28 days on Sauton's medium is 15.3%, a content which is similar to that of other mycobacteria.

The acetone-soluble fat is devoid of free phthiocol and contains 20.8% of mixed free fatty acids and 76% of neutral fat which can be fractionated by chromatography into four major fractions. These results are similar to those obtained by Aebi, Asselineau, and Lederer (1) for *Mycobacterium tuberculosis* var. *Brévannes*.

The A waxes of *Mycobacterium marianum* are very weakly acid and devoid of free acid of the mycolic type, a result which agrees with that reported by Asselineau (7) for the A waxes of the BCG strain. On chromatography of these waxes, two major fractions were obtained; a white neutral solid (M.P. 44°–48° C.) and a white solid of low acidity (M.P. 37°–44° C.). On saponification, the A waxes yielded 100% of ether-soluble fraction, 55.4% of which is insoluble in methanol. The methanol-soluble fraction contains a neutral fraction (4.6%) besides a mixture of fatty acids (22.7%). The methanol-insoluble fraction, a white solid with a M.P. of 58.5°–61° C., was repeatedly chromatographed on alumina and seven acid fractions were finally obtained. These fractions are probably mixtures of acids of the mycolic type and fraction III ac M, a white solid with a M.P. of 55.5°–57° C. and a T.M.W. of 1319, would agree very well with the formula $C_{88}H_{176}O_4$ (mol. wt. 1296) as proposed by Lederer (15) for some types of mycolic acids. The combined fractions I ac M, IV ac M, V ac M, and VII ac M would give a tentative average molecular weight of 905, which might be attributed to acids of the mycolic type with approximately 60 carbon atoms. Such hydroxy acids have already been isolated from *Mycobacterium tuberculosis* var. *Brévannes* by Asselineau (8). Fractions II ac M and VI ac M are probably mixtures of the above acids. Recrystallization of the neutral fraction obtained by saponification of the A waxes revealed two different compounds: one crystallizing from ethyl acetate as fine white needles with a M.P. of 56.5°–59.5 C., and the other, an amorphous white solid, with a M.P. of 38°–41° C.

Mycobacterium marianum contains 3.84% of phosphatides, a higher percentage than that reported in the literature for other strains of mycobacteria. Nevertheless, such a content agrees with the ones found in our laboratories for other mycobacteria, such as the BCG, H37Rv, and H37Ra strains. The phosphatides of *Mycobacterium marianum* were purified by filtration through cellulose powder columns and fractionated by chromatography on silicic acid – celite mixtures. Twelve different fractions were eventually obtained which differ one from another in their melting points, phosphorus content, and solubility in ether. Michel and Lederer (18) have already reported the fractionation of the phosphatides of *Mycobacterium marianum* into seven fractions. It is possible that some of our fractions, for instance B_1, C, and G, which have similar melting points and phosphorus contents, might be identical. The chemical analysis of each fraction is actually in progress and this will give a definite answer to this problem. Two of our fractions, D and E, are very heavily stained by Sudan black B. This may indicate a special chemical structure for these fractions. It must be noted that Michel and Lederer (18) have observed the presence of glucose and colamine in the phosphatides of *Mycobacterium marianum*; these compounds were never found in phosphatides of other mycobacteria.

Mycobacterium marianum has a very low content of chloroform extractable materials and, contrary to what is usually the case, a very small portion of these is made up of the D waxes. Furthermore, these D waxes have a low

melting point (38°–39.5° C.) whereas the D waxes of other mycobacteria are generally lipopolysaccharides of high melting points (about 200° C.). The D waxes of *Mycobacterium marianum* are only weakly stained by Sudan black B. The chloroform extracts contain a fraction very soluble in cold acetone in addition to the C waxes, which are insoluble in cold acetone. This soluble fraction was fractionated into a neutral white solid and a yellow oil.

Although the chemical analyses of the lipid fractions mentioned above have not yet been completed, the authors feel justified in saying that *Mycobacterium marianum* contains, as indicated by the cytochemical study, two acetone-insoluble fractions, the phosphatides and the D waxes, which have a special chemical composition. It may be concluded that the difference between a mycobacterium cultured *in vivo* and one cultured *in vitro* is not merely a quantitative one but a qualitative one as well, and this fact must be kept in mind in forthcoming studies.

Acknowledgment

The authors are indebted to Dr. Edith Mankiewicz for smears of tubercle bacilli from sputum. They also gratefully acknowledge the technical assistance of Miss Lidia Giampersa.

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A COMPARATIVE APPRAISAL OF THE PROPERTIES OF "STAPHYLOCOCCI" ISOLATED FROM CLINICAL SITES AND FROM DAIRY PRODUCTS[1]

By F. S. Thatcher and W. Simon

Abstract

A comparative study of properties of 263 isolates of mannitol-positive micrococci ("staphylococci") obtained from clinical sources and from cheese or butter has shown that isolates from the dairy products were predominantly type 42D, Group IV, of bovine origin, while among the isolates from hospital specimens Group III was most common, and type 42D was rarely encountered. Two strains lysed by phage type 42D have, however, been isolated from cases of severe enteritis developing after antibiotic therapy. Isolates from phage Group IV were more consistent than any other phage group in the production of coagulase, of phosphatase, and of potent amounts of alpha- and beta-hemolysins and enterotoxin. Except in Group IV, wide variation in these properties occurred within a species or within a phage group from either the dairy or clinical source.

Coagulase and phosphatase were not directly comparable in their indication of toxigenic strains and neither was absolute in relation to specific toxin production.

Toxins were detected in six species (after Bergey) though in only small amounts in all species other than *Micrococcus pyogenes*. Coagulase production was the more nearly consistent single indicator of toxigenicity among those tested.

The data are discussed in relation to pathogenicity of strains and food-poisoning propensities.

This paper reports a comparative study of properties of "staphylococci" isolated from clinical sites and from cheese and butter. The study was undertaken in order to establish for each of the two groups of cultures: (*a*) species relationships in accord with Bergey (2); (*b*) the production of specific toxins by individual isolates; (*c*) the production of coagulase and phosphatase, and the relationship of these properties to the ability of specific isolates to produce toxins; (*d*) the comparative ability of members of the various phage groups to produce coagulase, phosphatase, and toxins, and (*e*) to what degree staphylococci isolated from cheese or butter may contribute to infections in man as revealed by the occurrence of phage types common in these foods among hospitalized human patients.

Recent papers from this laboratory have reported that staphylococci are common in certain dairy products (20, 21) and that a substantial proportion of the staphylococcal cultures isolated from these foods were highly resistant to specific antibiotics (20). Further, extracts from specific cheese specimens and from culture filtrates of staphylococci isolated from cheese were shown to have contained fractions that were dermonecrotic, enterotoxic, and lethal to test animals. Hemolysins and enterotoxin have been demonstrated in preparations from both sources (21).

The comparison between the properties of strains producing coagulase and phosphatase was prompted by the observation of Barber and Kuper (1)

[1]*Manuscript received September 5, 1956.*
Contribution from the Microbiology Section, Laboratories of the Food and Drugs Directorate, Department of National Health and Welfare, Ottawa.

supported by White and Pickett (22), and by Carrère and Roux (5), that the production of phosphatase by staphylococci was a dependable indicator of pathogenicity, while many workers, particularly since the careful study by Haynes and Hucker (10), have given similar significance to the coagulase test. If valid, the phosphatase test used for such a purpose would offer advantages in simplicity and economy.

Methods

As basic criteria for isolation of "staphylococci" from cheese and butter, colonies were selected which grew readily and produced acid on a mannitol–peptone agar medium containing 8% sodium chloride, and which upon microscopic examination were found to consist of Gram-positive micrococci of cell diameter less than 1 micron. For comparative purposes, seven isolates of *Micrococcus epidermidis* (mannitol-negative) were also examined.

Cultures from clinical sites were forwarded from the laboratory of an Ottawa hospital. After learning that these had been preselected for coagulase production, some coagulase-negative micrococci from various infection sites were also obtained from the same hospital.

The properties used for species determination were those described in the key to the genus *Micrococcus* after Bergey (2), this particular scheme being based on the work of Hucker (11). The tests were carried out in accord with the Manual of Methods for Pure Culture Study of Bacteria (14), and were confirmed by a second series of tests. The reactions tested were: production of acid from mannitol, pigment formation, liquefaction of gelatin, behavior in litmus milk, growth on ammonium phosphate agar, and reduction of nitrate to nitrite.

The criterion for the production of coagulase was the formation within six hours at 37° C. of a firm gel in 0.5 ml. of rabbit plasma (Difco) after addition of the cells scraped from a "slant" culture on nutrient agar incubated at 37° C. for 24 hr.

Phosphatase was detected by the method of Barber and Kuper (1), which is based upon the demonstration of free phenolphthalein around a colony grown on nutrient agar containing phenolphthalein phosphate (previously tested for absence of the free indicator). After incubation of the test cultures for 48 hr. at 37° C., ammonia fumes were pumped into the tubed cultures with a hand pump. (The surface of the agar may alternatively be flooded with a dilute solution of ammonium hydroxide.) This caused any colony that had "split" the phosphate radicle from the colorless indicator complex to become surrounded by a red zone in the medium.

The production of toxins was determined from sterile filtrates prepared from cultures grown on Dolman's semisolid agar medium in an atmosphere of 30% CO_2 after incubation at 37° C. for 48 hr.

The presence of alpha- and beta-hemolysins in culture filtrates was determined by the methods described by Thatcher and Matheson (19) using washed sheep erythrocytes suspended in isotonic saline. Lysin titers were

expressed as the dilution that provided hemolysis of an estimated 50% of the blood cells, alpha-lysin being estimated after one hour at 37° C., and beta-lysin after a further incubation at 5° C. for one hour. Dermonecrotic factors were determined by the development of local ulceration in guinea pigs following the subcutaneous injection of 2 ml. of the sterile filtrate; enterotoxin was determined by intraperitoneal injection of 5 ml. of the filtrate after it had been boiled for 30 min. (to destroy any effective amount of lysins) into each of two cats, weighing from 1 to 3 kgm., that had been conditioned to laboratory life. Recurrent emesis from one of the two cats within from about 20 min. to 2 hr. was the initial criterion for enterotoxicity. Whenever only one cat reacted positively from a particular filtrate, a second test from a newly prepared filtrate was carried out. The occurrence of emesis from one of the second pair of cats was required before the presence of enterotoxin was recorded. The specimen for the emesis test was injected at a relatively high level in order to detect comparatively weakly-toxic filtrates (15). Delta-lysin was not determined because of previous failure to demonstrate any particular significance to this substance (12, 15).

Bacteriophage typing of cultures was carried out by standard procedures at the Phage Typing Centre of the Department of National Health and Welfare through the courtesy of Dr. E. T. Bynoe.

For comparative interest, the formation of toxins and phage group relationships were also determined from subcultures of well-known strains of staphylococci described in the literature, some of which had been in culture for many years.

Results

The distribution of species among 124 isolates of *Micrococcus* from cheese and butter, and 139 isolates from clinical sites, is listed in Table I. Ten species were recognized among the dairy isolates, six among the clinical cultures. The most common species in each group was *M. pyogenes*, which comprised 32% and 95% of the cultures from the two respective sources. The latter is probably a "weighted" value because of the selection, at the contributing hospital, of coagulase-positive strains during most of the period of collection of cultures. The varieties *aureus* and *albus* of *M. pyogenes* occurred in about equal proportions from each respective source.

The distribution of our total collection of cultures within the species described by Bergey (2) and their ability to produce coagulase and/or phosphatase is listed in Table II. Of 82 cultures of *M. pyogenes* var. *aureus*, 70 provided positive reactions for both enzymes; nine (clinical) were phosphatase-positive but coagulase-negative; three were negative for both. Of the 91 isolates classified as *M. pyogenes* var. *albus*, 30 gave the respective reactions for coagulase and phosphatase in the + + category, 39 in the − +, four in the + −, and 18 in the − − category. No other species provided a positive coagulase test, though phosphatase was detected in representatives of the species *aurantiacus*, *candidus*, *freudenreichii*, *flavus*, *caseolyticus*, and

TABLE I

THE DISTRIBUTION OF *Micrococcus* SPECIES FROM CLINICAL SITES AND FROM CHEESE* AND BUTTER*

Micrococcus species (after Bergey VI)	Determinative reactions						No. of cultures		% of cultures	
	Mannitol	Pigment	Gelatin	Litmus milk	Ammonium phosphate	Nitrite	Dairy	Clinical	Dairy	Clinical
pyogenes v. *aureus*	+	Orange	+	Acid, coag.	−	+	19	63	15.3	45.4
pyogenes v. *albus*	+	White	+	Acid, coag.	−	+	21	70	16.9	50.4
aurantiacus	+	Orange	−	Acid only	−	+	23	1	18.5	0.7
candidus	±	White	−	Acid only	−	−	22	1	17.8	0.7
freudenreichii	±	White	+	Acid, coag.	+	−	10	0	8.1	0
conglomeratus	±	Yellow	+	Acid only	+	+	4	1	3.2	0.7
caseolyticus	+	Variable	+	Acid, coag., pept.	+	−	5	0	4.1	0
flavus	±	Yellow	+	Acid, coag.	+	−	12	2	9.7	1.4
varians	+	Variable	−	Acid, coag. ±	+	+	1	0	0.8	0
ureae	+	White	−	N.C., alk.	+	−	1	0	0.8	0
epidermidis	−	White	−	Acid only	−	+	6	1	4.8	0.7
					Total isolates		124	139		

**Isolated with the aid of mannitol–salt agar (Difco).*

TABLE II

COAGULASE AND PHOSPHATASE PRODUCTION IN RELATION TO SPECIES FROM 263 ISOLATES OF MANNITOL-POSITIVE MICROCOCCI FROM CLINICAL AND DAIRY SOURCES

Species	No. of cultures		Reaction group	
	Clinical	Dairy	Coagulase	Phosphatase
M. pyogenes	133	40		
pyogenes var. *aureus*	53	17	+	+
	9	0	−	+
	1	2	−	−
pyogenes var. *albus*	26	4	+	+
	31	8	−	+
	2	2	+	−
	11	7	−	−
aurantiacus	0	4	−	+
	1	19	−	−
candidus	1	5	−	+
	0	17	−	−
freudenreichii	0	2	−	+
	0	8	−	−
conglomeratus	1	4	−	−
caseolyticus	0	1	−	+
	0	4	−	−
flavus	2	1	−	+
	0	11	−	−
varians	0	1	−	−
ureae	0	1	−	−
*epidermidis**	1	2	−	+
	0	4	−	−
	139	124		

**Mannitol-negative.*

epidermidis. The less frequent production of coagulase by the representatives of the *albus* variety as compared with the *aureus* variety of *M. pyogenes* will be noted.

Because of the diverse coagulase–phosphatase relationships shown by these cultures, a total of 50 of them were randomly chosen from the four groups having coagulase and phosphatase reactions in the respective combinations ++, −+, +−, −−, and determinations were made of their respective ability to produce alpha- and beta-hemolysins and enterotoxin. The data in Table III illustrates these properties in relation to source of the specific cultures.

TABLE III

THE RELATIONSHIP BETWEEN COAGULASE, PHOSPHATASE, AND SPECIFIC TOXIN PRODUCTION BY MICROCOCCI† ISOLATED FROM CLINICAL SITES AND FROM DAIRY PRODUCTS

No. of cultures						
Dairy	Clinical	Coagulase	Phosphatase	Alpha-lysin	Beta-lysin	Enterotoxin
8	4	+	+	+	+	+
0	7	+	+	+	−	+
0	1	+	+	+	+	−
0	3	+	+	+	−	−
0	1	−	+	+	+	+
1	2	−	+	+	−	+
2	0	−	+	−	−	+
7	2	−	+	−	−	−
1	0	+	−	+	+	+
0	1	+	−	−	−	−
0	1	−	−	+ (1 : 2)*	−	+
1	0	−	−	+ (1 : 2)	+ (1 : 4)	−
2	0	−	−	+ (1 : 8)	−	−
5	1	−	−	−	−	−

**Maximum titer.*

†*Selected randomly from 263 cultures grouped according to respective patterns of coagulase and phosphatase production.*

Of the 23 cultures in the + + coagulase–phosphatase category, all were *M. pyogenes*, of which 12 produced all three toxins. Eight of these 12 cultures originated from dairy products. Seven clinical cultures produced alpha-lysin and enterotoxin but no beta-lysin, three provided alpha-lysin alone, one produced alpha- and beta-lysins but no enterotoxin.

Of the 15 cultures that were coagulase-negative but phosphatase-positive, one clinical culture produced all three toxins (these relationships have been confirmed on two later occasions), three produced alpha-lysin and enterotoxin but no beta-lysin, two yielded enterotoxin but no lysins, while nine gave no indication of either of the three toxins.

Only two cultures were found that were coagulase-positive but phosphatase-negative. One of these (from cheese) produced all three toxins, the other (clinical) produced none of them.

The 10 cultures tested from the coagulase-negative and phosphatase-negative group provided one culture (clinical) that caused emesis in cats and also produced a trace of alpha-lysin; two others produced alpha-lysin to a titer of 1:8, traces of both alpha- and beta-lysins (to 1:4) were developed by another, while six gave no indication of toxigenicity.

Except for the fact that each of the dairy cultures that were positive for both coagulase and phosphatase produced all three toxins, the data in Table III shows wide variability in toxin production among the cultures whether they were from dairy or clinical sources.

TABLE IV

TOXIN PRODUCTION BY MICROCOCCI IN RELATION TO SPECIES*

Species	Alpha-lysin	Dermonecro-toxin	Beta-lysin	Enterotoxin	No. of cultures
M. pyogenes var. *aureus*	+	+	+	+	6
	+	+	−	+	5
	+	+	+	−	1
	+	+	−	−	2
	+	−	+	−	1
M. pyogenes var. *albus*	+	+	+	+	5
	+	+	−	+	2
	+	+	−	−	1
	+	−	+	+	2
	+	−	−	+	2
	+	−	+	−	1
	−	−	−	+	1
	−	−	−	−	6
M. aurantiacus	+‡	−	−	+	1
	+‡	+	−	−	1
	−	−	−	−	4
M. flavus	−	−	−	−	3
M. candidus	−	−	−	−	2
M. freudenreichii	+‡	−	−	+	1
M. conglomeratus	+†	−	−	−	1
M. epidermidis	−	−	−	+	1
	−	−	−	−	1

*Selected in accord with varying patterns of phosphatase and coagulase production (see Table II).
†Maximum titer 1 : 8.
‡Maximum titer 1 : 4.

Table IV illustrates the species relationships of the 50 cultures selected, as described earlier, in accord with their coagulase–phosphatase properties. Among the representatives of the species *M. pyogenes* all possible combinations of toxin production are evident. None of the *aureus* strains of this species were entirely without toxin. Six *albus* strains produced no toxin; one produced enterotoxin alone; one produced alpha- and beta-lysin but no enterotoxin. Of the 16 isolates producing beta-lysin, 13 also produced enterotoxin; of the 32 that produced alpha-lysin, 24 of them yielded enterotoxin.

Traces of alpha-lysin were produced by three species other than *pyogenes*, namely, *aurantiacus*, *freudenreichii*, and *conglomeratus*. In one instance, only, was the toxin titer from these last cultures sufficiently high to cause dermonecrosis. Representatives of the three species *aurantiacus*, *freudenreichii*, and *epidermidis* provided enough enterotoxin to evoke emesis in cats.

The distribution of the clinical and dairy cultures among standard phage groups is shown in Table V. As has been commonly reported (3, 4, 7, 16), clinical isolates belonged predominately to Group III. Group IV was not represented. Of the dairy cultures in this particular collection (obtained from widely dispersed origins) 80% were of Group IV and 20% untypable by

TABLE V

THE PHAGE GROUPING OF TOXIGENIC MICROCOCCI ISOLATED FROM CLINICAL SITES AND FROM DAIRY PRODUCTS*

Source of cultures	Distribution within phage groups (%)					
	Gp. 1	Gp. 2	Gp. 3	Gp. 4	Gp. M	Untypable†
Clinical‡	10.5	10.5	47.4	0	31.6	21
Dairy	0	0	0	80	0	20

**Selected randomly from within collections of cultures with varying patterns of coagulase and phosphatase production.*
†With standard phages.
‡Some isolates reacted to phage types within more than one group, hence the sum of percentages exceeds 100.

standard phages. Twenty-one per cent of the clinical specimens were also in this last category. It should be recalled that only coagulase-positive strains are typable by standard phages.

Table VI reports the production of enterotoxin and the range in specific lysin titers from toxigenic representatives of the selected isolates described above. Of particular interest are the consistently high titers of both alpha- and beta-lysins and the invariable production of enterotoxin from the representatives of phage Group IV. Their capacity to produce potent amounts of beta-lysin is outstanding. Otherwise, the data in this table show no particular relationship between phage group and lysin production. Only two of our typed cultures proved to be members of Group I. This small number would not warrant any generalization from the fact that these two failed to produce enterotoxin.

TABLE VI

THE RANGE IN TITER OF SPECIFIC LYSINS AND THE PRODUCTION OF ENTEROTOXIN BY TOXIGENIC* MICROCOCCI IN RELATION TO PHAGE GROUPING

Phage group	No. cultures	Lysin titer		Enterotoxin
		Alpha	Beta	
1/M	2	1 : 4 – 1 : 128	1 : 8 – 1 : 128	–
2	2	1 : 32 – 1 : 128	1 : 128	+
3	7	1 : 32 – 1 : 1028	1 : 64 – 1 : 1028	+ (6/7)
3/M	2	1 : 64 – 1 : 128	1 : 64 – 1 : 128	±†
4	8	1 : 512 – 1 : 8192	1 : 2048 – 1 : 131,072	+
M	2	1 : 64 – 1 : 128	1 : 64 – 1 : 128	±
Untypable	8	1 : 2 – 1 : 16	1 : 2 – 1 : 32	±

**The toxigenic specimens among the 50 selected randomly in accord with varying patterns of coagulase and phosphatase production.*
†Production variable.

A compilation of phage types that produce specific lysins and enterotoxin (from among the selected group) is shown in Table VII. Toxigenicity and hemolysin production seem to bear little relationship to phage specificity,

TABLE VII

THE RELATIONSHIP BETWEEN PHAGE TYPE AND SPECIFIC TOXIN PRODUCTION BY *Micrococcus* SPECIES

Toxin produced	Phage types	Phage groups
Alpha-lysin	3A/3B/3C/6/7/31/42B/42C/42D/42E/44/47/47B/47C/51/52A/53/54/55/70/73/75/76/77/81	1/2/3/4/M
Beta-lysin	3A/3C/7/31/42D/44/47/51/52A/53/54/70/75/76/77	1/2/3/4/M
Enterotoxin	3A/3B/3C/6/7/42B/42C/42D/42E/47/47B/47C/51/53/54/55/70/73/75/76/77/81	2/3/4/M
Enterotoxin consistently absent	31/44/52A	1

except that the only phage types among those listed in Table VII that consistently were associated with failure to produce enterotoxin were 31, 44, and 52A, all of which are of Group I. The earlier comment about inadequate numbers applies to this observation also.

The specific toxin production and phage relationships of subcultures of strains described in the literature are indicated in Table VIII. A broad variation in toxin production is evident, while two well documented food-poisoning strains (230 and 12069 alpha) as now extant in our laboratory were coagulase-negative and untypable, though still capable of producing their characteristic toxins.

TABLE VIII

THE PRESENT TOXIN AND PHAGE RELATIONSHIPS OF SPECIFIC STRAINS OF STAPHYLOCOCCI DESCRIBED IN EARLIER LITERATURE

Strain identification	Reference	Toxin production					Phage group
		α-Lysin	β-Lysin	δ-Lysin	Enterotoxin	Coagulase	
J 32 A	Surgalla (17)	−	+	−	+*	+	2
230	Casman (6)	Trace	−	−	+	+	3
S 6	Surgalla (17)	+	−	Trace	+	+	3
F & D 7	Levi *et al.* (13)	−	−	−	−	+	3
S 1	Surgalla (17)	+	+	?	+	−	Untypable
12069-α	Dolman *et al.* (8)	+	−	−	+	−	Untypable
L 16	Thatcher *et al.* (19)	+	+	+	+	+	4

**Not normally considered enterotoxic; see Thatcher and Matheson (19) for experimental evidence regarding our particular subculture.*

Discussion

The foregoing data suggest a number of conclusions in relation to the primary objectives of this paper.

The mannitol-positive micrococci isolated, respectively, from dairy products and from clinical sources showed a comparable range in taxonomic characters, and in the production of coagulase, phosphatase, alpha- and beta-hemolysins, and enterotoxin.

Representatives of the species *M. pyogenes* occurred in the higher proportion among the clinical isolates, but within the species *M. pyogenes* the varieties *aureus* and *albus* occurred in roughly equal proportions among isolates from each source. Strains of *M. pyogenes* var. *aureus* from the dairy products were, however, more consistent producers of alpha- and beta-hemolysins and of enterotoxin, and yielded a higher potency of each toxin than did the clinical strains. All of the randomly selected strains from the dairy products were lysed by phage type 42D and were therefore members of phage Group IV, considered to be of bovine origin (7). Representatives of this phage group were not found among the 139 specimens from the Ottawa hospital. This would suggest that the staphylococci in cheese or butter appear to be of numerically minor significance as a cause of serious infection in man. Bynoe (4) and Brown *et al.* (3) have shown that members of phage Group IV occur among staphylococci isolated from hospitalized patients in Canada at a proportion of less than 1%. However, the capacity of strains of type 42D to produce high titers of toxins is of interest in relation to the fact that two cultures among a collection of staphylococci, isolated from cases of severe enteritis that developed as a sequel to antibiotic therapy, were lysed by phage type 42D. The bovine forms have also been established as able to cause food poisoning in man (18).

The so-called indicators of pathogenicity or of toxigenicity, coagulase and phosphatase, were not directly comparable in their association with toxin production. Among the strains tested, coagulase was the more dependable indicator of the capacity to produce one of the three toxins considered in this study, since nine cultures produced phosphatase without indication of either of the three toxins, while this was true for only one coagulase-positive strain. The production of coagulase was confined to the species *M. pyogenes*, whereas phosphatase was produced by representatives of this and six other species. Conversely, a small proportion of cultures that failed to produce either coagulase or phosphatase, nevertheless did produce toxins. Thus, neither enzyme is absolute in its toxin association.

Variations in the ability of a specific culture to produce coagulase have been described recently by Fusillo and Jaffurs (9) and by Lack and Wailling (12). Observations in this laboratory concur. Three cultures of antibiotic-resistant toxigenic staphylococci from clinical sources were coagulase-negative on three separate tests shortly after isolation, but after five months of routine transfer were shown to be coagulase-positive members of phage Group III.

Two of these cultures caused emesis in monkeys.[2] Our particular subculture of strain 12069 alpha of Dolman—a beta-lysin-free mutant from a typical golden, hemolytic strain of *M. pyogenes*—is coagulase-negative and untypable with standard phages but still produces alpha-lysin and enterotoxin.

Our total data thus suggests that no single character among those investigated in this study provides a fully dependable indication of a toxigenic strain.

The sparse occurrence in hospitals of the potently toxigenic Group IV strains when compared with the frequency of occurrence of Group III strains suggests that the former do not have comparable propensities for causing illness to man except through food poisoning or under such exceptional circumstances of enteritis developing as a sequel to prolonged antibiotic therapy. Further study will be undertaken before any generalization is deduced.

Lack and Wailling (12) showed that some 7% of coagulase-positive cultures isolated from clinical sites failed to produce toxins. Our results were comparable. Dolman *et al.* and Haynes and Hucker (10) have previously referred to such occurrences.

Our findings that enterotoxin may be produced by coagulase-negative strains of *M. pyogenes*, indeed from other species of *Micrococcus*, points to the need for investigators of food poisoning to be aware of this fact, even though such a relationship may be encountered very infrequently. The prevailing practice of ascribing only to coagulase-positive strains any propensity for causing food poisoning would seem to be at variance with the indication from the work of Haynes and Hucker (10), who demonstrated the occurrence of enterotoxin-producing strains among six species of *Micrococcus*. Two of these species and two others were represented among the enterotoxin-producing strains in the present study.

A quantitative relationship between the emetic reaction in cats and in man needs to be established in order to determine the minimal significant potency of enterotoxin as revealed by cats that can cause illness in man. Since the preparation of this manuscript, it has been established that three of five cultures that were weakly enterotoxic to cats were not toxic to monkeys when tested on one occasion with culture filtrates prepared in another laboratory.[3]

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[2]*Tested through the courtesy of Dr. C. E. Evans, American Meat Institute, Chicago.*
[3]*Through the courtesy of Dr. C. E. Evans, American Meat Institute, Chicago.*

Discussion

The foregoing data suggest a number of conclusions in relation to the primary objectives of this paper.

The mannitol-positive micrococci isolated, respectively, from dairy products and from clinical sources showed a comparable range in taxonomic characters, and in the production of coagulase, phosphatase, alpha- and beta-hemolysins, and enterotoxin.

Representatives of the species *M. pyogenes* occurred in the higher proportion among the clinical isolates, but within the species *M. pyogenes* the varieties *aureus* and *albus* occurred in roughly equal proportions among isolates from each source. Strains of *M. pyogenes* var. *aureus* from the dairy products were, however, more consistent producers of alpha- and beta-hemolysins and of enterotoxin, and yielded a higher potency of each toxin than did the clinical strains. All of the randomly selected strains from the dairy products were lysed by phage type 42D and were therefore members of phage Group IV, considered to be of bovine origin (7). Representatives of this phage group were not found among the 139 specimens from the Ottawa hospital. This would suggest that the staphylococci in cheese or butter appear to be of numerically minor significance as a cause of serious infection in man. Bynoe (4) and Brown *et al.* (3) have shown that members of phage Group IV occur among staphylococci isolated from hospitalized patients in Canada at a proportion of less than 1%. However, the capacity of strains of type 42D to produce high titers of toxins is of interest in relation to the fact that two cultures among a collection of staphylococci, isolated from cases of severe enteritis that developed as a sequel to antibiotic therapy, were lysed by phage type 42D. The bovine forms have also been established as able to cause food poisoning in man (18).

The so-called indicators of pathogenicity or of toxigenicity, coagulase and phosphatase, were not directly comparable in their association with toxin production. Among the strains tested, coagulase was the more dependable indicator of the capacity to produce one of the three toxins considered in this study, since nine cultures produced phosphatase without indication of either of the three toxins, while this was true for only one coagulase-positive strain. The production of coagulase was confined to the species *M. pyogenes*, whereas phosphatase was produced by representatives of this and six other species. Conversely, a small proportion of cultures that failed to produce either coagulase or phosphatase, nevertheless did produce toxins. Thus, neither enzyme is absolute in its toxin association.

Variations in the ability of a specific culture to produce coagulase have been described recently by Fusillo and Jaffurs (9) and by Lack and Wailling (12). Observations in this laboratory concur. Three cultures of antibiotic-resistant toxigenic staphylococci from clinical sources were coagulase-negative on three separate tests shortly after isolation, but after five months of routine transfer were shown to be coagulase-positive members of phage Group III.

Two of these cultures caused emesis in monkeys.[2] Our particular subculture of strain 12069 alpha of Dolman—a beta-lysin-free mutant from a typical golden, hemolytic strain of *M. pyogenes*—is coagulase-negative and untypable with standard phages but still produces alpha-lysin and enterotoxin.

Our total data thus suggests that no single character among those investigated in this study provides a fully dependable indication of a toxigenic strain.

The sparse occurrence in hospitals of the potently toxigenic Group IV strains when compared with the frequency of occurrence of Group III strains suggests that the former do not have comparable propensities for causing illness to man except through food poisoning or under such exceptional circumstances of enteritis developing as a sequel to prolonged antibiotic therapy. Further study will be undertaken before any generalization is deduced.

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A SURVEY OF FUNGAL POLYSACCHARIDES[1]

By S. M. Martin and G. A. Adams

Abstract

A survey of the constituents of the polysaccharides of 31 species of fungi (12 genera) has been made. On the basis of two of the constituents, glucuronic acid and fucose, the extracellular preparations could be classified into three groups. *Mucor* and *Rhizopus* species produced material containing glucuronic acid and fucose, in addition to galactose, glucose, and mannose. *Fusarium* and *Trichoderma* species yielded glucuronic acid but not fucose and the remainder yielded neither glucuronic acid nor fucose. The intracellular preparations could be classified into two groups on the basis of the presence or absence of fucose. Those from *Mucor* and *Rhizopus* species yielded fucose whereas the remainder did not. Glucosamine occurred in all intracellular preparations and glucuronic acid was absent.

Introduction

Few, if any, surveys have been made of the sugar constituents of the polysaccharide fractions from a variety of fungi although many reports deal with the isolation and characterization of individual polysaccharides. For example, a polygalactose and a polyglucose were isolated from *Aspergillus niger* (4) and a polygalactose and a polymannose were isolated from *Penicillium charlesii* (2). Varianose, a polysaccharide containing glucose, galactose, and idose or altrose, was isolated from *Penicillium varians* (5). Capreolinose, a polysaccharide containing glucose, galactose, mannose, and malonic acid was isolated from *Penicillium capreolinum* (3). Recently Hough and Perry (6) isolated, from *Mucor racemosus*, a polysaccharide containing fucose, galactose, mannose, and glucuronic acid. Since it appeared that such a survey might reveal avenues for future research in the fields of carbohydrate chemistry and mold physiology, we undertook to determine the constituents of the crude polysaccharide fractions of a heterogeneous group of fungi. The present communication reports the results of this survey.

Methods

Cultural Conditions

The 31 species of fungi used in this survey were selected as representing taxonomically a rather heterogeneous group and without reference to their polysaccharide forming ability. All of the *Mucor* and *Rhizopus* species were obtained from Dr. J. W. Groves, Botany and Plant Pathology Division, Science Service, Canada Department of Agriculture, and the remainder from Miss M. T. Clement, Division of Applied Biology, National Research Laboratories. The taxonomic position of the test organisms is shown in Fig. 1.

[1]*Manuscript received September 13, 1956.*
Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, Canada. Issued as N. R. C. No. 4140.

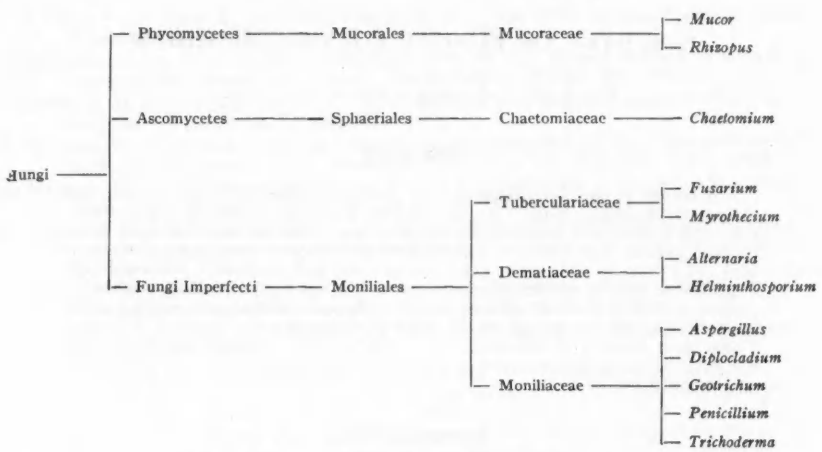

FIG. 1. Taxonomic position of the fungi tested.

All organisms were grown under similar conditions of medium, temperature, and degree of aeration, no attempt being made to obtain maximum yields of polysaccharides. Cultures were grown at 28° C. on malt agar slants (1.25% malt syrup, 2% agar) until well sporulated. A water suspension of spores was then used to inoculate 3-liter Fernbach flasks containing 500 ml. of fermentation medium (glucose, 5%; NH_4NO_3, 0.2%; $K_2HPO_4 \cdot 3H_2O$, 0.15%; KH_2PO_4, 0.19%; $MgSO_4 \cdot 7H_2O$, 0.05%; plus growth factors at one-eighth concentration used by McDonald and Gibbons (7)). The cultures were harvested after incubation at 28° C. on a rotary shaker for 14 days or when about 80–100% of the sugar had been utilized, whichever was the shorter.

Recovery of Polysaccharides

The medium was separated from the mycelium by filtration, concentrated about 20-fold under reduced pressure, poured into five volumes of ethanol, and allowed to stand overnight. The precipitate was separated by decantation, dissolved in water, and autoclaved for five minutes (to destroy cellulolytic enzymes which in some instances were capable of destroying the cellophane sac used for dialysis). The solution was then dialyzed overnight against running water, reconcentrated, and poured into ethanol. The precipitate was separated by centrifugation, washed with ethanol and ether, dried *in vacuo*, and weighed. This constituted the "extracellular polysaccharide". The absence of free sugars in this material was established chromatographically.

The mycelium was washed repeatedly with water, dried *in vacuo*, and weighed. The dried mold pad was then broken up, wetted with water, and extracted for six to eight hours with methanol in a Soxhlet extractor. The extracted mycelium, after being dried and ground to a fine powder, constituted the "intracellular polysaccharide".

Analysis for Sugar Components

A portion of the extracellular fraction was hydrolyzed for 16 hr. at 100° C. with *N* sulphuric acid in a sealed tube. The hydrolyzate was then neutralized with barium carbonate, centrifuged, and chromatographed on Whatman No. 1 paper with pyridine – ethyl acetate – water (1 : 2 : 1) as the solvent. *p*-Anisidine hydrochloride (3% in ethanol) (1) was used as the spray reagent. An aliquot of the "intracellular polysaccharide" was triturated with 72% sulphuric acid for three hours at room temperature, diluted with water to a final concentration of 1*N* sulphuric acid, and heated at 100° C. for four hours in a pressure bottle. After neutralization and concentration, the hydrolyzate was chromatographed.

Analysis for Glucuronic Acid

As glucuronic acid was expected as a constituent of at least some of the preparations (6), a simple and rapid method for its detection, such as chromatography or electrophoresis, was essential. However, this acid is difficult to detect chromatographically, especially in the presence of mixtures of sugars and partial hydrolysis products. Also the acid readily forms a lactone in acid solution and thus, after the hydrolysis, behaves as a neutral component on paper electrophoresis, the normal neutralization procedure being insufficient to open the lactone ring. It was noted, however, that when a salt of the acid was run on paper electrophoresis, at pH 7.0 or slightly higher, in the presence of sugars or of the lactone, a clear separation was obtained. The equilibrium is so strong in favor of lactone formation that somewhat drastic conditions are required to complete the neutralization. To avoid the possible effect of strong alkali on the sugars present, $BaCO_3$ was selected as the neutralizing agent. To effect the conversion of the lactone to the salt a solution containing glucuronolactone was added to a considerable excess of barium carbonate, sealed in a tube, and heated for 10 hr. at 75° C. This treatment also allows differentiation between glucuronic and galacturonic acids for, although both free acids have the same mobility on paper, the latter does not form a lactone and runs without the barium carbonate treatment. The procedure was tested by chromatography and electrophoresis for the production of artifacts; none were observed.

As a routine procedure for the detection of glucuronic acid and glucosamine, the hydrolyzate, after prior chromatographic examination, was heated with barium carbonate as described above. The suspended material was then allowed to settle and the supernatant used to spot strips of Whatman 3 MM paper. The components were then separated on an "E-C" electrophoresis apparatus* using *M*/40 phosphate buffer at pH 7.1 and 900 v. (1.8 ma. per cm. breadth of paper) for 45 min. When dry, the strips were sprayed with *p*-anisidine hydrochloride and observed under white and ultraviolet light.

**E-C Apparatus Co., Swarthmore, Pa.*

Results and Discussion

The constituents of the extracellular polysaccharides from the 31 test organisms are shown in Table I. The polysaccharide preparations can be classified in three groups on the basis of the presence or absence of glucuronic acid and fucose: (1) contain glucuronic acid and fucose, (2) contain glucuronic acid but not fucose, (3) contain neither glucuronic acid nor fucose.

All of the *Mucor* and *Rhizopus* species produced polysaccharide material containing glucuronic acid, fucose, galactose, and mannose. In all preparations, except those from *Mucor adventitius* and *M. hiemalis*, small amounts of glucose were also found. Preparations from the latter two species yielded an unidentified reducing compound (X_1) which ran faster than ribose on chromatograms. A negatively charged reducing compound (X_2) which ran faster than glucuronic acid on electrophoresis was present in small amounts in some preparations. The inability to demonstrate X_2 conclusively in some was probably a matter of concentration. Compounds X_1 and X_2 were

TABLE I

CONSTITUENTS OF "EXTRACELLULAR POLYSACCHARIDE" PREPARATIONS

Species*	N.R.C. Culture No.	Glucuronic acid	Fucose	Glucose	Galactose	Mannose	Ribose	X_1	X_2	X_3	Number of experiments	Dry weight/culture, mgm.†‡
Mucor adventitius	M.101	+	+	−	+	+	−	+	+	−	1	95
M.hiemalis	M.92	+	+	−	+	+	−	+	+	−	1	95
M.javanicus	M.95	+	+	+	+	+	−	−	+	−	1	115
M.plumbeus	M.93	+	+	+	+	+	+	−	+	−	2	341
M.racemosus	M.96	+	+	+	+	+	−	−	+	−	1	176
M.spinosus	M.97	+	+	+	+	+	−	−	+	−	1	255
M.sylvaticus	M.102	+	+	+	+	+	−	−	±	−	2	251
Rhizopus oryzae	M.98	+	+	+	+	+	−	−	±	−	2	557
R.tamari	M.94	+	+	+	+	+	+	−	±	−	2	251
R.tonkinensis	M.100	+	+	+	+	+	+	−	±	−	2	346
Fusarium lini	9593	+	−	+	+	+	−	−	−	−	4	203
F.moniliforme	10052	+	−	+	+	+	−	−	−	−	4	132
F.sp.	F.41	+	−	+	+	+	−	−	−	−	4	192
F.sp.	F.68	+	−	+	+	+	−	−	−	−	4	296
Trichoderma viride	V.139	+	−	+	+	+	−	−	−	−	3	317
Alternaria tenuis	E.23	−	−	+	+	+	−	−	−	−	3	237
Aspergillus oryzae	A2.1	−	−	+	+	+	−	−	−	+	2	392
A.nidulans	A3.31	−	−	−	+	+	−	−	−	+	2	141
A.niger	A1.233	−	−	+	+	+	−	−	−	−	2	204
A.terreus	A.630	−	−	−	+	+	−	−	−	−	2	53
Chaetomium brasiliensis	117	−	−	+	+	+	−	−	−	−	2	36
C.globosum	V.159	−	−	+	+	+	−	−	−	−	2	65
Diplocladium sp.	V.145	−	−	+	+	+	−	−	−	−	2	61
Geotrichum candidum	22	−	−	+	+	+	+	−	−	−	3	530
Helminthosporium sp.	V.146	−	−	+	+	+	−	−	−	−	2	91
Myrothecium verrucaria	V.155	−	−	+	+	+	−	−	−	−	2	254
Penicillium claviforme	P.328	−	−	+	+	+	−	−	−	−	1	88
P.chrysogenum	P.357	−	−	+	+	+	−	−	−	−	1	168
P.griseo-fulvum	P.30	−	−	+	+	+	−	−	−	−	1	111
P.islandicum	P.15	−	−	+	+	+	−	−	−	−	1	1413
P.rugulosum	P.42	−	−	+	+	+	−	−	−	−	1	198

**Not arranged in taxonomic order.*
†Based on single experiments.
‡For fermentation time, dry weight of mold, and % sugar utilization see Table II.

presumed to be different as their occurrences did not parallel one another. Traces of ribose were detected in preparations from *Mucor plumbeus*, *Rhizopus tamari*, and *R. tonkinensis*, but it is suspected that this sugar may have arisen from nucleic acids. The presence of glucuronic acid, fucose, galactose, and mannose in all *Mucor* and *Rhizopus* preparations suggests that a polysaccharide similar to that described by Hough and Perry (6) is common to all members of these genera.

The extracellular fractions from two unrelated genera, *Fusarium* and *Trichoderma*, contained glucuronic acid, galactose, glucose, and mannose. Neither fucose nor X_2 was detected. Since fucose was undetected in these preparations it is evident that the polysaccharide described by Hough and Perry (6) was not produced by these organisms.

The remaining cultures produced polysaccharide material containing neither glucuronic acid nor fucose nor X_2. All yielded galactose, glucose, and mannose except those from *Aspergillus nidulans* and *A. ferreus* in both of which glucose was absent. A positively charged reducing compound (X_3) which ran faster than glucosamine on electrophoresis was detected in preparations from *Aspergillus nidulans* and *A. oryzae*. A small amount of ribose was found in one preparation from *Geotrichum candidum*.

On the basis of color intensities and spot areas it was noted that in general the "extracellular polysaccharides" contained relatively more galactose and mannose than glucose. Glucuronic acid and fucose, when present, were relatively abundant.

The constituents of the "intracellular polysaccharides" of the test organisms are shown in Table II. Here the preparations can be divided into two groups based on the presence or absence of fucose.

Like the "extracellular polysaccharides", the intracellular preparations of *Mucor* and *Rhizopus* species differed from all others in containing fucose. However neither glucuronic acid nor X_2 was detected. In all species glucose, mannose, and glucosamine were also present. The presence of galactose was established in three species but was somewhat doubtful in seven. Ribose was noted in *Mucor hiemalis* and *M. sylvaticus*.

All of the other species tested contained glucose, mannose, and glucosamine, with little or no galactose. An unidentified positively charged compound (X_4), perhaps the same as X_3, was found in the mycelium of *Aspergillus niger*.

In general the extracted mycelium of the test organisms contained much glucose and glucosamine, less mannose and very little galactose, a picture rather different from that found in the extracellular preparations. When present, fucose was about equivalent to mannose in concentration.

The data presented here should not be construed as establishing fixed patterns of polysaccharide constituents for these fungi since preliminary experiments indicated that the changing of growth conditions produced quantitative, if not qualitative, changes in the constituents of the preparations. Attempts to draw conclusions relative to individual polysaccharides present in the crude preparations are also unwise. However, this survey has indicated

TABLE II

CONSTITUENTS OF "INTRACELLULAR POLYSACCHARIDE" PREPARATIONS

Species*	Fucose	Glucose	Galactose	Mannose	Ribose	Glucosamine	X_4	Number of experiments	Dry weight of mold/culture, gm. ‡,§	Fermentation time, days†	Sugar utilization, %†
Mucor adventitius	+	+	+	+	±	+	−	1	1.75	5	98
M.hiemalis	+	+	+	+	−	+	−	1	2.80	5	98
M.javanicus	+	+	+	+	−	+	−	1	2.80	7	80
M.plumbeus	+	+	+	+	−	+	−	1	1.84	14	60
M.racemosus	+	+	+	+	−	+	−	1	1.68	7	80
M.spinosus	+	+	+	+	−	+	−	1	3.02	10	84
M.sylvaticus	+	+	+	+	−	+	−	1	2.08	14	44
Rhizopus oryzae	+	+	±	+	−	+	−	1	0.47	12	88
R.tamari	+	+	±	+	−	+	−	1	1.34	14	64
R.tonkinensis	+	+	±	+	−	+	−	1	1.72	14	64
Fusarium lini	−	+	+	+	−	+	−	3	6.55	8	100
F.moniliforme	−	+	±	+	−	+	−	3	2.21	7	92
*F.*sp. (41)	−	+	±	+	−	+	−	3	5.01	7	91
*F.*sp. (68)	−	+	±	+	−	+	−	3	5.83	7	86
Trichoderma viride	−	+	+	+	±	+	−	1	1.86	14	62
Alternaria tenuis	−	+	+	+	−	+	−	1	5.67	14	86
Aspergillus oryzae	−	+	+	+	−	+	−	1	3.70	7	100
A.nidulans	−	+	+	+	−	+	−	1	8.91	7	76
A.niger	−	+	+	+	−	+	+	1	3.71	7	86
A.terreus	−	+	+	+	−	+	−	1	4.84	7	84
Chaetomium braziliensis	−	+	+	+	−	+	−	1	2.73	14	47
C.globosum	−	+	+	+	−	+	−	1	0.73	14	5
Diplocladium sp.	−	+	+	+	−	+	−	1	0.13	14	2
Geotrichum candidum	−	+	+	+	±	+	−	1	1.62	12	100
Helminthosporium sp.	−	+	+	+	−	+	−	1	2.80	14	26
Myrothecium verrucaria	−	+	+	+	−	+	−	1	3.03	14	50
Penicillium claviforme	−	+	+	+	−	+	−	1	0.80	14	24
P.chrysogenum	−	+	+	+	−	+	−	1	3.07	7	100
P.griseo-fulvum	−	+	+	+	−	+	−	1	3.54	7	98
P.islandicum	−	+	+	+	−	+	−	1	3.65	7	90
P.rugulosum	−	+	+	+	−	+	−	1	6.27	9	64

**For culture number see Table I.*
†Based on single experiment.
‡Dry weight of mycelium before extraction.
§For weight of extracellular polysaccharide see Table I.

that hitherto undescribed polysaccharides are undoubtedly present in these organisms. Preliminary studies have also indicated the presence, in the ethanol soluble fraction, of oligosaccharides, some of which probably have not yet been characterized. The survey has also suggested a number of problems in the field of mold physiology. For example, a study of the mode of formation of polysaccharides may be simplified because many of the polysaccharides are extracellular and contain monomers, such as glucuronic acid and fucose, which might act as markers in following synthesis. It was also observed that several of the *Mucor* species contained cellulolytic enzymes and these may be concerned with the breakdown of the extracellular polysaccharides.

Acknowledgment

The authors wish to thank Dr. J. W. Groves and Miss M. T. Clement for cultures received and Mr. A. E. Castagne and Mr. G. D. Powers for their technical assistance.

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THE TRICARBOXYLIC ACID CYCLE IN PSEUDOMONAS NIGRIFACIENS[1]

By J. Robinson[2] and H. Katznelson

Abstract

All the enzymes of the tricarboxylic acid cycle, with the exception of oxalosuccinic acid decarboxylase, were detected in sonic extracts of the moderately halophilic bacterium *Pseudomonas nigrifaciens*. Several of the component enzymes of the cycle exhibited a slight salt requirement; activity of the others was reduced in the presence of added salt. These results are in contrast to the optimal salt requirement of the organism for growth (1.5 to 3.0% NaCl).

Apart from the effects of salt on their activities, the component enzymes of the tricarboxylic acid cycle in *P. nigrifaciens* appear to be similar to those described from other sources.

Introduction

Isolated enzyme systems have been studied and identified in several halophilic bacteria (1, 16) but no attempt has been made to study enzymes catalyzing a series of reactions in these organisms. The tricarboxylic acid cycle has been shown to operate in a variety of tissues and organisms including avian erythrocytes (17), tissues from plants (2), and bacteria (3, 6). The present report concerns the tricarboxylic acid cycle in the moderately halophilic *Pseudomonas nigrifaciens*, an organism causing surface discoloration of salted print butter (8).

Materials and Methods

P. nigrifaciens was cultured in Fernbach flasks in a medium containing casein hydrolyzate (Difco Casamino Acids) 6.7 gm.; sodium chloride, 15.0 gm.; salt solutions A[3] and B[4], 5.0 ml. each; malic acid, 6.7 gm., or sodium succinate, 10.0 gm.; water, 1000 ml.; pH 7.0. The cultures were incubated 18 hr. at 27° C. on a rotary shaker, the cells harvested, washed three times with 1.5% sodium chloride solution, and standardized to a uniform turbidity of 600 with the blue filter in the Klett–Summerson colorimeter. To prepare cell-free extracts the cells were centrifuged from saline solution, the precipitate resuspended in a volume of water equivalent to twice the weight of sedimented cells, and the resulting suspension treated for two minutes in a Raytheon 10 kc. sonic oscillator. After centrifugation, the supernatant fluid was found to contain approximately 25.0 mgm. protein per ml. as determined by the method of *Lowry et al.* (9). Oxygen uptake and CO_2 evolution were measured in a conventional Warburg apparatus at 30° C. Citric acid was determined by the method outlined by Natelson, Pincus, and Lugovoy (11) as modified by

[1]*Manuscript received August 27, 1956.*
Contribution No. 424 from the Bacteriology Division, Science Service, Canada Department of Agriculture, Ottawa, Ontario.
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[3]*Salt solution A* = K_2HPO_4 *and* KH_2PO_4, *25.0 gm. each, in 250 ml. solution.*
[4]*Salt solution B* = $MgSO_4 \cdot 7H_2O$, *10.0 gm.;* *NaCl*, $FeSO_4 \cdot 7H_2O$ *and* $MnSO_4 \cdot 4H_2O$, *0.5 gm. each, in 250 ml. solution.*

Eisenberg (6). Reduction of DPN[5] and TPN was determined spectrophotometrically at 340 mμ (2). Formation of α-ketoglutaric acid was evaluated quantitatively by the method of Friedman and Haugen (7). "Keto-" acids were separated as the phenylhydrazones and identified by the chromatographic procedure of Cavallini, Frontali, and Toschi (4).

Transacetylase was prepared from *Escherichia coli* by a modification of the method described by Ramakrishnan and Martin (15). *E. coli*, N.R.C. strain 482, was obtained from Dr. S. M. Martin, Division of Applied Biology, National Research Council, Ottawa, and was used for the production of this enzyme. Cell-free extracts of the lyophilized preparations of *E. coli* suspended in water were prepared by treating the suspension for 20 min. in the Raytheon sonic oscillator.

ATP (99% purity), CoA (75% purity), DPN (95% purity), DPT, oxaloacetic, α-ketoglutaric, fumaric, and *l*-malic acids were obtained from Nutritional Biochemicals Corporation; TPN "80" from the Sigma Chemical Company; *cis*-aconitic acid (anhydride) from the California Foundation for Biochemical Research; and glyoxylic acid from the Bios Laboratories. Acetyl phosphate was obtained from Dr. B. B. Migicovsky, Chemistry Division, Science Service, Canada Department of Agriculture, Ottawa, and was purified by alcoholic precipitation from a concentrated aqueous solution.

Results

Intact cells of *P. nigrifaciens* oxidized some of the intermediates of the tricarboxylic acid cycle, namely: succinic, fumaric, and oxaloacetic acids. Citric, *cis*-aconitic, isocitric, α-ketoglutaric, and malic acids were not oxidized to any appreciable extent. Only one intermediate, succinic acid, was oxidized readily by sonic extracts of the test organism (Table I).

TABLE I

OXYGEN UPTAKE BY INTACT CELLS AND SONIC EXTRACTS OF *Pseudomonas nigrifaciens* WITH VARIOUS SUBSTRATES (RESULTS EXPRESSED AS μL. OF OXYGEN CONSUMED DURING A FOUR-HOUR PERIOD; ENDOGENOUS VALUES SUBTRACTED)

Substrate	Intact cells (10 μM. substrate)	Sonic extract (20 μM. substrate)
Citric	6.4	92.3
cis-Aconitic	7.8	21.2
Isocitric	10.1	0
α-Ketoglutaric	29.5	9.2
Succinic	412.3	345.7
Fumaric	302.3	0
Malic	90.2	6.6
Oxaloacetic	166.0	0
Pyruvic	644.8	0
Glutamic	444.8	52.6
Glyoxylic	83.2	0
Methylglyoxal	0	0

[5]*Abbreviations used: CoA, coenzyme A; TPN, triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; DPT, diphosphothiamine; ATP, adenosine triphosphate; TCA, trichloroacetic acid.*

There is no indication from these results that a tricarboxylic acid cycle necessarily is operative in this organism. However, activity of a condensing enzyme was demonstrated in sonic extracts by determining citric acid formation in reaction mixtures containing oxaloacetic acid, acetyl phosphate, CoA, and a source of transacetylase (Table II). This system is similar to that used by Ochoa, Stern, and Schneider (13).

Results presented in Fig. 1 show that the isocitric dehydrogenase of *P. nigrifaciens* was TPN- but not DPN-linked. These results were confirmed

TABLE II

ACTIVITY OF CONDENSING ENZYME IN SONIC EXTRACTS OF *P. nigrifaciens*

Additions to reaction mixture (0.5 ml. each)	Citric acid produced (μM.)
Transacetylase (sonic extract of *E. coli*)	0.45
"Condensing enzyme" (sonic extract of *P. nigrifaciens*)	0
Transacetylase and "Condensing enzyme"	4.05

NOTE: *Experimental conditions: 2.0 ml. 0.1 M potassium phosphate buffer, pH 7.5; 0.1 ml. 0.1 M cysteine, pH 7.0; 0.2 ml. 0.1 M sodium oxaloacetate; 0.2 ml. of a solution of acetyl phosphate adjusted to pH 7.0 with NaOH; 0.05 ml. 0.005 M CoA, pH 7.0; sonic extract additions as noted; volume adjusted to 3.55 ml. with water; incubated 120 min. at 27° C.; 1.0 ml. 10% TCA added to stop the reaction.*

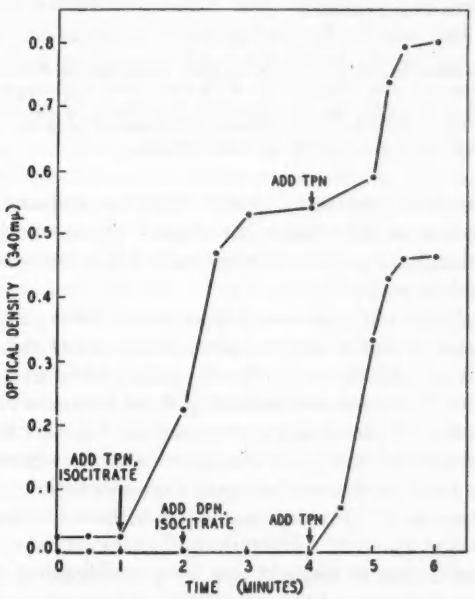


FIG. 1. Activity of isocitric dehydrogenase in sonic extracts of *P. nigrifaciens*. Cuvettes contained 1.5 ml. 0.1 *M* phosphate buffer, pH 7.5, with 1.0% NaCl, 1.0 ml. water, 0.1 ml. sonic extract, and 0.1 ml. 0.005 *M* $MnCl_2$; 0.2 mgm. TPN; 0.132 mgm. DPN; and 0.1 ml. 0.05 *M* sodium isocitrate added as required. No untoward reaction of manganese and phosphate was noted in this medium.

by measuring the formation of α-ketoglutaric acid from citric, *cis*-aconitic, or isocitric acids in the presence and absence of DPN, TPN, and $MnCl_2$. Glyoxylic acid could not be identified as a product of the reaction. Formation of α-ketoglutaric acid from citric or *cis*-aconitic acids also indicates the presence of aconitase.

α-Ketoglutaric acid was utilized by sonic extracts of *P. nigrifaciens* in the presence of DPN, TPN, DPT, and ATP but not in their absence. Enzyme activity was determined by the ferricyanide technique of Quastel and Wheatley (14). The results, presented in Table III, show that 4.67 μM. of α-keto-

TABLE III

α-KETOGLUTARIC DEHYDROGENASE ACTIVITY IN SONIC EXTRACTS OF *P. nigrifaciens* IN THE PRESENCE OF ADDED COFACTORS

Cofactor addenda	CO_2 (μM.)	Residual α-ketoglutaric acid (μM.)
None	0	9.94
DPN	0	
DPN, TPN	0	
DPN, TPN, DPT	0	
DPN, TPN, DPT, ATP	5.1	5.27
μM. α-ketoglutaric acid oxidized		4.67

NOTE: *Experimental conditions: Each Warburg vessel contained 0.1 ml. 3.3% $NaHCO_3$, 0.1 ml. cofactor addenda (0.066 mgm. DPN, 0.1 mgm. TPN, 0.126 mgm. DPT, 1.18 mgm. ATP), and water to 2.2 ml. The side arms contained respectively 0.2 ml. water or α-ketoglutaric acid, pH 7.0, in 0.2 ml. and 0.2 ml. freshly prepared ferricyanide reagent. Incubated 100 min. at 30° C. in an atmosphere containing 5% CO_2 and 95% N_2.*

glutaric acid were used. Although 5.1 μM. CO_2 were produced simultaneously, little significance can be attached to this figure because both α-ketoglutaric acid and succinic acid, a product of the oxidation of α-ketoglutaric acid, react with ferricyanide to produce CO_2.

Succinic acid was the only intermediate in the tricarboxylic acid cycle whose oxidation was linked directly with oxygen. Oxidation of this compound may also be followed anaerobically by the ferricyanide procedure. Oxygen uptake of sonic extracts of *P. nigrifaciens* from 20 μM. of succinic acid was plotted as a function of time and the results presented in Fig. 2. Reasonably close agreement exists between the actual volume of oxygen consumed and that expected on the basis of a one step oxidation of succinic to fumaric acid. Malic acid accumulated in the reaction mixture without changing the apparent stoichiometry of the reaction. Fumaric and malic acids were identified as products of the oxidation of succinic acid by a modification of the chromatographic procedure of Lugg and Overell (10).

Results presented in Figs. 3-A to 3-F inclusive show that cell-free extracts of *P. nigrifaciens*, dialyzed two hours against water, reduced DPN and TPN in the presence of malic acid. The reduction of DPN was inhibited (Fig. 3-A)

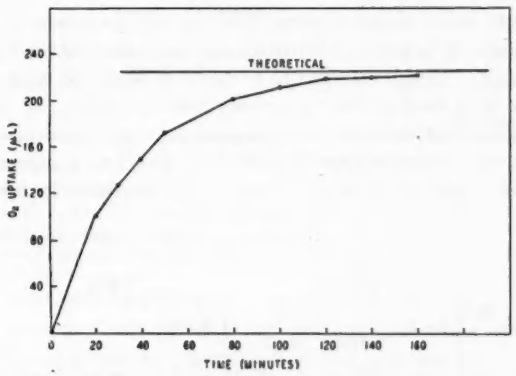


FIG. 2. Succinoxidase in sonic extracts of *P. nigrifaciens*. Warburg vessels contained 1.5 ml. 0.1 *M* phosphate buffer, pH 7.5; 0.5 ml. sonic extract; and 0.8 ml. water. Side arm contained 0.2 ml. water or 20 μM. sodium succinate. Inset: 0.2 ml. 10% KOH. Incubated in air at 30° C. Endogenous values subtracted.

but that of TPN was stimulated (Fig. 3-B) by $MnSO_4$. Glutamic acid increased the extent of reduction of DPN (Fig. 3-C) but had no effect on that of TPN (Fig. 3-D). Both DPN (Fig. 3-E) and TPN (Fig. 3-F) were reduced at pH 9.0, but no reduction of DPN and only a limited reduction of TPN could be observed at pH 7.0. The products of the reaction which required TPN were separated as the phenylhydrazones and identified as pyruvic and oxaloacetic acid.

Preparations of the test organism which were able to reduce TPN in the presence of malic acid were able to reduce this coenzyme in the presence of fumaric acid at pH 9.0 and also in the presence of the products of the oxidation of succinic acid adjusted to pH 9.0. These results suggest that succinic acid is oxidized stepwise by way of fumaric and malic acids to oxaloacetic acid.

Oxaloacetic decarboxylase was determined anaerobically with freshly prepared oxaloacetic acid, pH 6.0. The nonenzymatic decarboxylation of this compound was determined with a preparation of *P. nigrifaciens* heated in boiling water for five minutes. Residual oxaloacetic acid was determined by the aniline–citrate procedure (5). Results presented in Table IV show that 6.3 μM. of oxaloacetic acid decomposed spontaneously in 100 min. and 12.6 μM. were decarboxylated in the presence of sonic extracts of the test organism; thus 6.3 μM. were decarboxylated as a result of enzyme action.

P. nigrifaciens is a moderately halophilic organism which requires between 1.5% and 3.0% NaCl for optimal development (8). The results presented in Fig. 4 show that the ability of intact cells of the test organism to oxidize succinic, fumaric, and pyruvic acids was affected by salt. Oxygen uptake of intact cells of *P. nigrifaciens* from these substances was greatest in the presence of 1.5% NaCl. The response to NaCl of the enzymes of the tricarboxylic acid cycle in sonic extracts of *P. nigrifaciens* was then determined. The results, presented in Fig. 5, show that activity of all the enzyme systems of

the cycle which were tested was reduced in the presence of 1.5% NaCl. Maximal activity of isocitric dehydrogenase was evident in the presence of 0.5 to 1.0% NaCl. Results similar to these were obtained with α-ketoglutaric dehydrogenase and oxaloacetic decarboxylase. Activity of the condensing enzyme, succinoxidase, succinic dehydrogenase, and aconitase was reduced measurably as the concentration of sodium chloride was increased from 0 to 4.0%. The malic dehydrogenase was extremely sensitive to even 1.0% NaCl.

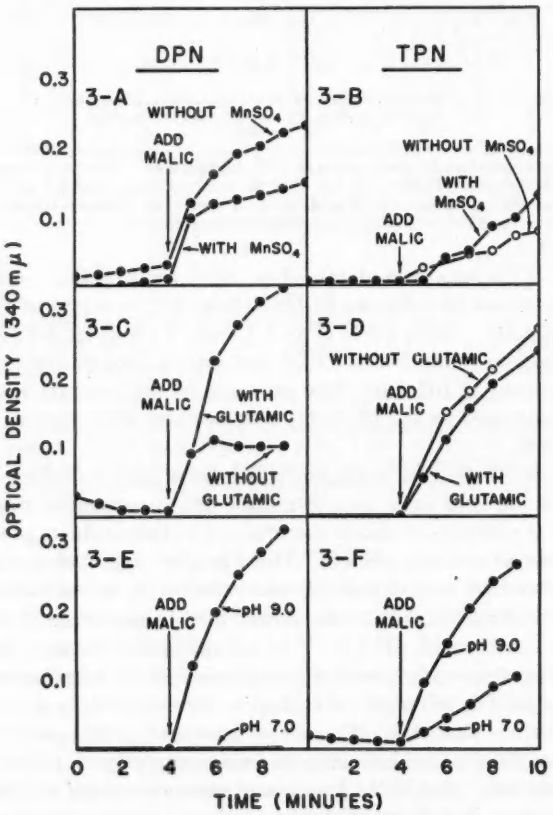


FIG. 3-A to 3-F. Reduction of DPN and TPN by malic dehydrogenase and "malic enzyme" from sonic extracts of *P. nigrifaciens* dialyzed two hours against water.

A. Effect of $MnSO_4$ on the reduction of DPN.
B. Effect of $MnSO_4$ on the reduction of TPN.
C. Effect of glutamic acid on the reduction of DPN.
D. Effect of glutamic acid on the reduction of TPN.
E. Effect of pH on the reduction of DPN.
F. Effect of pH on the reduction of TPN.

Cuvettes contained 1.5 ml. 0.1 *M* phosphate glycine buffer, pH 9.0, unless specified otherwise, and 0.1 ml. of enzyme. Sodium glutamate, 20 μM.; *l*-malate, 5 μM.; $MnSO_4$, 0.05 μM.; TPN, 0.2 mgm.; and DPN, 0.132 mgm. added as required and the volume adjusted to 3.0 ml. with water.

TABLE IV

OXALOACETIC ACID DECARBOXYLASE ACTIVITY OF SONIC EXTRACTS OF *P. nigrifaciens*

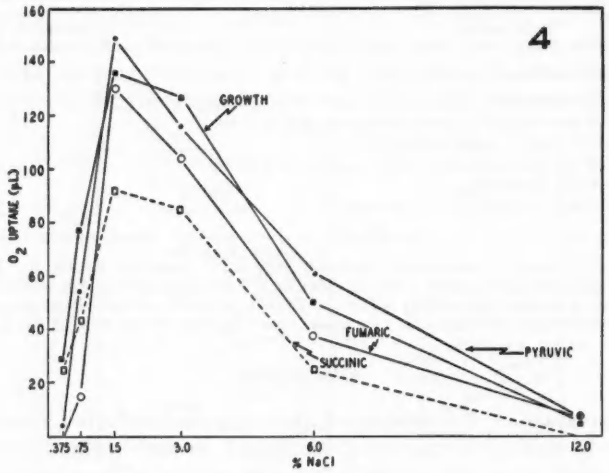
Treatments	Oxaloacetic acid (μM.)
Initial level of oxaloacetic acid	12.5
Final level of oxaloacetic acid	Nil
CO_2 liberated enzymatically and nonenzymatically from oxaloacetic acid by sonic extracts	12.6
CO_2 liberated nonenzymatically from oxaloacetic acid by heated sonic extracts	6.3
CO_2 liberated enzymatically (by difference)	6.2

NOTE: *Experimental conditions: Each Warburg vessel contained 1.5 ml. 0.1 M potassium phosphate buffer, pH 6.0; 0.1 ml. 0.001 M $MnCl_2$; 0.5 ml. sonic extract of P. nigrifaciens. The two side arms contained respectively 0.2 ml. of freshly prepared aniline-citrate reagent or 3.0 N H_2SO_4 and oxaloacetic acid, pH 6.0. Incubated anaerobically for 100 min. at 30° C.*

Discussion

Determination of the activity of the component enzyme systems of the tricarboxylic acid cycle may be accomplished, in part at least, by evaluating oxygen uptake from the various substrates with or without added cofactors (2, 18). In the present work, stimulation of oxygen uptake could be induced only by adding succinic acid to sonic extracts or by adding succinic, oxaloacetic, and pyruvic acids to intact cells of *P. nigrifaciens;* the other members of the tricarboxylic acid cycle were not oxidized directly by either sonic extracts or intact cells. Activity of these enzymes was detected in sonic extracts by several indirect methods, including measurement of citric and α-ketoglutaric acid formation, TPN reduction in the presence of isocitric and fumaric acids, DPN and TPN reduction in the presence of malic acid, and oxaloacetic acid formation and decarboxylation. All enzymes of the tricarboxylic acid cycle, with the exception of oxalosuccinic decarboxylase, were detected by such procedures and appear to be similar to those described from plant, animal, and microbial sources. Several of the component enzyme systems of the cycle exhibited a slight salt requirement and in this respect may differ somewhat from those described previously; activity of the other enzyme systems of the cycle was reduced in the presence of added salt. The latter observations are in contrast to the optimal salt requirement of the organism for growth.

That both a "malic enzyme" and a malic dehydrogenase are present in sonic extracts of *P. nigrifaciens* is indicated by the observation that the reduction of TPN but not DPN is catalyzed by $MnSO_4$ and that the rate of reduction of DPN but not TPN is increased by the addition of glutamic acid. The malic enzyme isolated from liver homogenates by Ochoa, Mehler, and Kornberg (12) was catalyzed by TPN and $MnSO_4$. Oxaloacetic acid has been reported (6) to be an effective inhibitor of malic dehydrogenase activity. Eisenberg (6) and Rubinstein and Denstedt (17) were able to minimize the effects of oxaloacetic acid by including cyanide with the reactants. Similar



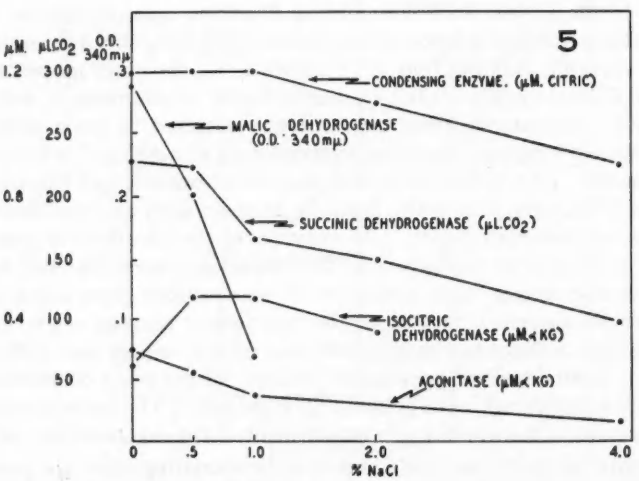


FIG. 4. Effect of NaCl on the growth in nutrient broth and on the oxygen uptake of intact cells of *P. nigrifaciens* from 10 µM. of succinic, fumaric, and pyruvic acids, pH 7.0, in 0.1 *M* phosphate buffer, pH 7.0. Incubated 60 min. at 30° C.; endogenous values subtracted.

FIG. 5. Effect of NaCl on the activity of specific enzyme systems in sonic extracts of *P. nigrifaciens*.

results were obtained with the malic dehydrogenase from *P. nigrifaciens* using glutamic acid instead of cyanide. The glutamic–aspartic transaminase, which was present in sonic extracts of the test organism, presumably removed the oxaloacetic acid from solution following addition of the glutamic acid. Optimal activity of the "malic enzyme" and the malic dehydrogenase from animal sources is evident at pH 7.4, while that of similar enzymes from the moderately halophilic pseudomonad is evident at pH 9.0. That malic acid may be oxidized by several mechanisms with different pH and coenzyme requirements is substantiated by the observations of Brummond and Burris (2), who described a malic dehydrogenase from lupine seedlings which was catalyzed equally well by TPN and DPN at pH 7.0, and Campbell and Smith (3), who described a similar enzyme from *Pseudomonas aeruginosa* which was catalyzed by TPN alone at pH 8.6.

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THE EFFECT OF DIETARY ENTEROCOCCI AND CHLORTETRACYCLINE HYDROCHLORIDE ON THE INTESTINAL FLORA AND GROWTH OF CHICKS[1]

By G. W. Anderson,[2] M. M. Hauser,[2] M. L. Wright,[2] and J. R. Couch[3]

Abstract

The addition to the diet daily of 10% (v/w) of a high level (28×10^9 cells) of a viable culture consisting of two strains of beta hemolytic enterococci sensitive to 10 and 30 μgm./ml. of chlortetracycline HCl respectively caused a highly significant reduction in growth of chicks. The reduction in growth was accompanied by an increase in numbers of enterococci in the ceca and a decrease in coliforms in the ileum and ceca as well as a decrease in lactobacilli in all segments of the intestine examined. The addition of 40 gm. of crystalline chlortetracycline HCl to the diet overcame the reduction in growth caused by the high level of dietary enterococci and resulted in a decrease in numbers of enterococci in the ileum and ceca.

Introduction

Several investigators have shown that the inclusion of an antibiotic or a combination of antibiotics, either in a crude or pure form, in the rations of young chicks and/or poults results in improved growth. A complete review of the literature on the possible mechanisms by which antibiotics stimulate growth has been published by Branion *et al.* (3). It showed that the only common denominator of all the antibiotics was the effect they have on the intestinal flora or on the medium in which they exist.

The possible ways in which an antibiotic may favorably affect the intestinal flora and hence promote growth may be listed as follows:

(1) The antibiotics may inhibit or destroy organisms which produce subclinical infections, that is, they suppress organisms which produce toxic reactions and cause a slowing or depression of growth of the host animal.

(2) Antibiotics may produce an increase in the number or activity of organisms which synthesize certain known or unknown vitamins or growth factors which are eventually made available to the host.

(3) Antibiotics inhibit organisms which compete with the host for available nutrients.

Sufficient evidence is available to support all three possibilities. However, owing to the diversity of methods for analyses there is much variance in the intestinal microfloral picture. Nevertheless, an excellent review by Finland (6)

[1]*Manuscript received July 19, 1956.*
Contribution from the Department of Bacteriology, Ontario Agricultural College, Guelph, Ontario, and the Department of Biochemistry and Nutrition, Texas Agricultural and Mechanical College, College Station, Texas. Part of a thesis submitted by the senior author, in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Presented in part at the Fifth Annual Meeting of Canadian Society of Microbiologists, Macdonald College, St. Anne de Bellevue, Quebec.
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of the bacteriological findings of workers in the field of antibiotics and intestinal microflora indicated that there was, generally, an increase in the *coli–aerogenes* group and a reduction in enterococci.

Anderson *et al.* (2), who fed an atypical *Escherichia coli*, and Romoser *et al.* (10), who fed *Aerobacter aerogenes* to chicks, both reported increases in growth accompanied by an increase in intestinal coliforms and a reduction in enterococci. As a result of these findings, this experiment was designed to determine the effect of feeding enterococci which had been reduced in numbers in the intestine by the feeding of an antibiotic. It was felt that the feeding of such a group of organisms might further aid in explaining the theory of antibiotic growth promotion.

Methods

The experiment involved five duplicate groups of 22 Columbian Rock male chicks randomized according to weight. The birds were reared in electrically heated battery brooders with raised wire floors. The experimental diets and tap water were supplied ad libitum during the four week duration of the trial. The basal diet contained 10% ground wheat, 42% ground yellow corn, 30% soybean oil meal (50% protein), 2% dehydrated green feed, 2.5% meat meal, 3% fish meal, 2.5% dried buttermilk, 5% tallow, 1% ground limestone, 2% steamed bone meal, 0.25% iodized salt. In addition 11 gm. vitamin A supplement (10,000 I.U./gm.), 23 gm. vitamin D_3 supplement (1500 I.C.U./gm.), 11 gm. DL-methionine, 45 gm. choline chloride (25%), 9 gm. manganese sulphate (feed grade, 65%), 4 gm. riboflavin supplement No. 54 (1 gm./oz.), 2 gm. calcium pantothenate supplement No. 52.S (2 gm./oz.), 1 gm. niacin, 23 gm. 3-nitro-4-hydroxyphenyl arsonic acid premix (10%), and 23 gm. vitamin B_{12} supplement (6 mgm./lb.) were mixed with each 100 lb. of diet. To this basal diet was added a low and high level of bacterial culture. It was also fed in combination with the high level of culture and 40 gm. per ton of crystalline chlortetracycline hydrochloride.

The mixed enterococcus cultures used for feeding were isolated from the cecal contents of chicks fed a practical starter diet. These cultures were maintained on "S.F." broth of Hajna and Perry (8). Forty-eight-hour-old cultures were centrifuged and the cells washed three times in tap water. The final cell suspensions were mixed in the feed daily at a 10% level (v/w). The number of viable cells fed each day was estimated by the M.P.N. method. An attempt was made to maintain the following number of cells per milliliter of suspension; low level 140×10^4 and high level at 28×10^8. These levels of mixed cultures supplied 0.51 and 1.0% dry matter respectively.

Cultural and physiological tests were carried out employing standard methods on the mixed enterococcus culture used in the feeding trials. Serological characteristics of the cultures were determined using 13 antisera and the ring test method of Lancefield (9). Slide agglutination tests were conducted with the same antisera. The antibiotic sensitivity of the types of organisms comprising the dietary mixed enterococcus culture was

determined by the tube dilution method (12). The concentrations of crystalline chlortetracycline hydrochloride ranged from 0 to 200 μgm. per ml. For growth and feed consumption studies, the birds were weighed individually at weekly intervals. After four weeks on these diets, five birds were sacrificed from each group and bacteriological analyses were made on the pooled contents of the following segments of the intestinal tract, duodenum, ileum, and ceca. The methods of Anderson *et al.* (1) were used for the determinations.

Results

Cultural examination indicated that the mixed enterococcus culture consisted of three strains or types of organisms, two of which were apparently similar. All three types were typical Gram-positive spheres with an average diameter of 0.8 μ, found singly or in small clusters. They were nonmotile, nonsporogenous, and without capsules when cultured in "S.F." broth. All types were micro-aerophilic.

Physiological results (Table I) indicate that cultures 5 and 11 have the same physiological characteristics and would appear to be the same organism. This is substantiated by the serological data (Table II), which show the reaction of each organism tested with 13 Lancefield grouping antisera by the slide agglutination and ring test methods. All three types, while positive in the ring test to C antisera, required three to five minutes to appear and remained for at least three hours.

TABLE I

PHYSIOLOGIC CHARACTERISTICS OF THE THREE ORGANISMS IN THE MIXED DIETARY ENTEROCOCCI CULTURE

Test	Culture Number		
	4	5	11
Hemolysis (beta)	+	+	+
Growth at 40° C.	+	+	+
Resists heat at 60° C. for 30 min.	+	+	+
Growth in 6% NaCl	+	+	+
Growth at pH 7.6	+	+	+
Growth at pH 9.6	+	−	−
Methylene blue (0.1%)	+	+	+
Sodium hippurate	+	−	−
Litmus milk	+RC	−	−
Starch hydrolysis	−	−	−
Gelatin	−	−	−
Lactose	+	+	+
Mannitol	+	−	−
Glucose	+	+	+
Trehalose	+	+	+
Sorbitol	+	−	−

NOTE: *R* = *reduction of dye.*
C = *coagulation.*
\+ = *growth.*

TABLE II

SEROLOGIC CHARACTERISTICS OF THE THREE ORGANISMS IN THE DIETARY MIXED ENTEROCOCCI CULTURE

Culture Number	Test	Antisera Lancefield groups												
		A	B	C	D	E	F	G	H	K	L	M	N	O
4	A_1	−	−	−	±	±	±	−	±	−	−	−	++	−
	B_1	−	−	+	−	−	−	−	−	−	−	−	−	−
5	A_1	++	±	−	±	++	±	±	++	−	++	−	++	++
	B_1	−	−	+	−	−	−	−	−	−	−	−	−	−
11	A_1	++	±	−	±	++	±	±	++	−	++	−	++	++
	B_1	−	−	+	−	−	−	−	−	−	−	−	−	−

NOTE: A_1 = *Slide agglutination test.*
B_1 = *Ring test.*

TABLE III

CHLORTETRACYCLINE HCl SENSITIVITY TESTS OF THE THREE ORGANISMS IN THE DIETARY MIXED ENTEROCOCCI CULTURE

Culture number	Concentration of chlortetracycline HCl (μgm./ml.)						
	2	5	10	15	20	25	30
4	+	+	−	−	−	−	−
5	+	+	+	+	+	+	−
11	+	+	+	+	+	+	−

NOTE: + = *not sensitive, growth.*
− = *sensitive, no growth.*

The slide agglutination test on the other hand showed considerable heteroagglutinability (Table II). However it did serve to differentiate culture 4 from 5 and 11 as did the physiological tests. Haemagglutination tests gave equally confusing results, which would indicate that additional antigens should be searched for before the serological identity of such strains is determined.

Chlortetracycline HCl sensitivity (Table III) of the enterococci making up the dietary mixed culture was recorded as the lowest concentration completely inhibiting growth of the organisms after 24 hr. incubation at 37° C. The data indicate that culture 4 was inhibited by 10 μgm./ml. of chlortetracycline HCl while cultures 5 and 11 were inhibited by 30 μgm./ml. of the antibiotic.

The weight and feed efficiency data (Table IV) for the four week trial period were analyzed by the "*t*" test (11). They indicate that the feeding of 10% (v/w) of the high level of the viable mixed enterococcus culture caused a highly significant reduction in growth, as may be seen when the mean weight of the birds of group 4 is compared to that of group 1 (birds on the basal diet). The addition of the low level of the culture caused a slight but not significant reduction in weight. The addition of 40 gm. of crystalline chlortetracycline HCl per ton of feed to the diets of groups 2 and 5 resulted in an improvement in weight when compared to the basal diet, group 1. In the case of group 2 the improvement in weight was highly significant. However when group 5 was compared to the group 4, which received the high level of culture alone,

TABLE IV

THE EFFECT OF DIETARY ENTEROCOCCI AND CHLORTETRACYCLINE HCl ON THE GROWTH OF CHICKS

Group No.	Additions to basal diet		Weekly results for 4 weeks Av. wt. (gm.) 1	2	3	4	Feed/gain 4th week
1	None	R_1*	63.6 (21)	137.0	236.2	357.5 (21)	2.09
		R_2	68.2 (22)	122.3	217.2	346.3 (21)	2.13
		Av.	65.9 (43)	129.6	226.7	351.9 (42)	2.11
2	Chlortetracycline HCl	R_1	84.1 (22)	174.4	302.8	428.0 (22)	1.95
		R_2	89.0 (22)	163.6	288.4	417.1 (22)	1.96
		Av.	86.5 (44)	169.0	295.6	422.5 (44)	1.95
3	Enterococci low level	R_1	65.0 (22)	144.6	217.3	332.1 (22)	2.25
		R_2	61.0 (22)	129.2	200.1	315.1 (22)	2.31
		Av.	63.0 (44)	136.9	208.7	323.6(44)	2.28
4	Enterococci high level	R_1	38.1 (22)	90.1	165.1	268.0 (21)	3.05
		R_2	54.1 (22)	109.2	186.1	272.0 (21)	2.99
		Av.	46.1 (44)	99.6	175.6	270.0 (42)	3.02
5	Enterococci high level + chlortetracycline HCl	R_1	68.4 (22)	153.5	243.3	382.4 (22)	2.08
		R_2	74.2 (22)	141.1	233.5	367.6 (22)	2.10
		Av.	71.3 (44)	147.3	238.4	375.0 (44)	2.09

NOTE: ( ) = *Number birds surviving in each group.*
*R** = *Replicate.*

the increase in weight due to the presence of chlortetracycline HCl was found to be highly significant. A slight improvement was observed when group 5 was compared to group 1, birds on the basal diet. The effect of treatment on the efficiency of feed utilization paralleled the results obtained for improvement in weight.

The effect of feeding enterococcus cultures and chlortetracycline HCl on the numbers of bacteria in the contents of different segments of the intestinal tract are shown in Table V. All values are expressed on the basis of wet

TABLE V

THE EFFECT OF CHLORTETRACYCLINE HCl AND DIETARY ENTEROCOCCI ON THE INTESTINAL BACTERIA OF CHICKS AT FOUR WEEKS OF AGE

Group	Segment	Log no. of bacteria per gram of intestinal contents (wet wt.) Total count	Coliforms	Lactobacilli	Enterococci
1. Control	Duodenum	5.01	<5.00	5.31	<5.00
	Ileum	5.63	5.39	6.65	5.30
	Ceca	9.44	7.71	6.78	9.32
2. Chlortetracycline HCl	Duodenum	5.85	<5.00	<5.00	<5.00
	Ileum	5.89	5.41	6.28	<5.00
	Ceca	9.32	7.60	5.47	7.60
3. Low level culture	Duodenum	4.91	<5.00	5.30	<5.00
	Ileum	5.00	5.20	7.11	5.78
	Ceca	9.44	7.14	5.54	9.20
4. High level culture	Duodenum	5.04	<5.00	5.04	<5.00
	Ileum	5.72	<5.00	5.87	5.30
	Ceca	10.11	6.85	5.04	10.73
5. High level culture + chlortetracycline HCl	Duodenum	4.96	<5.00	5.01	<5.00
	Ileum	6.92	5.47	7.11	4.60
	Ceca	9.90	7.26	5.32	7.72

weight of the contents of the various segments of the intestine. This was necessary because young chicks often had insufficient amounts of intestinal solids present to make microbiological determinations. It was observed that the bacterial counts of all organisms except the lactobacilli increased from the upper to lower segments of the intestinal tract. The feeding of the high level of the cultures, group 4, resulted in an increase in numbers of enterococci, accompanied by a decrease in both coliforms and lactobacilli. This increase in enterococci was reflected by an increase in total numbers of viable organisms. The addition of the low level of the cultures to the diet, group 3, did not noticeably affect the numbers of bacteria of any of the groups when compared to those of the control group. However, the inclusion of 40 gm. of chlortetracycline HCl per ton of feed as in groups 2 and 5 resulted in a reduction in numbers of enterococci and lactobacilli as compared to the numbers in the control group, while on the other hand there was no apparent difference in the numbers of coliform organisms.

Discussion

The evidence obtained in this work indicated that the feeding of 10% (v/w) of a high level (28×10^9 cells) of mixed enterococcus cultures caused a highly significant reduction in weight. The mixed culture consisted of three types or strains of organisms, two of which were apparently similar. This was substantiated by cultural, physiological, serological, and chlortetracycline HCl sensitivity tests. Cultural and physiological tests of these organisms showed that they resembled in some respects members of the enterococcus group described by Dubos (5). However, owing to variation in their serological types it was difficult to relate them directly to any of the previously described members of the enterococcus group (9). The three cultures were weakly beta hemolytic, and both the ring and slide agglutination tests showed considerable heteroagglutinability; however they aided in differentiating culture 4 from 5 and 11. It can be seen that there is still a certain amount of confusion as to complete typing of these enterococci isolated from the intestinal tract of chicks. It indicates that more complete methods are required and that additional antigens should be searched for before the serological identity of such strains can be certain. The fact that inhibition of growth was obtained by dietary enterococci at a high level and that this growth inhibition was overcome by feeding 40 gm. of chlortetracycline HCl per ton of feed is further evidence of the growth-promoting effect of the antibiotic. It also aids in explaining the importance of the intestinal microflora in the role of increased growth following the use of antibiotics. The feeding of enterococci at a sufficiently high level resulted in an increase in numbers of enterococci and a decrease in coliforms and lactobacilli, which was accompanied by a highly significant reduction in growth and feed efficiency. However, when chlortetracycline HCl was included in the diet, growth inhibition was removed and the number of enterococci was reduced. The results (Table V) would indicate that apparently the action, in part, of the

antibiotic in this experiment was to cause a decrease in enterococci and lactobacilli and to tend to maintain the coliform count at approximately that of the control group. These results appear to be in keeping with the results of Guzman-Garcia *et al.* (7), Cook *et al.* (4), and Anderson *et al.* (2); the latter indicated that the maintaining of a high number of coliforms was due to the increase of an atypical strain of *E. coli*. The above results substantiate the fact that there is a relationship of bacterial groups to one another in the intestinal tract which can be altered by dietary changes. The reduction of growth caused by dietary enterococci would be due probably to the competition of such organisms and host for normal nutrients, or the production of some undesirable cellular products which place the host under "stress" conditions. Whatever may be the case, results of this experiment indicate that the lack of growth caused by enterococci can be overcome by feeding chlortetracycline HCl.

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CARBOHYDRATE METABOLISM BY LEUCONOSTOC MESENTEROIDES[1]

By A. C. Blackwood and E. R. Blakley

Abstract

Sixty-six carbohydrates were surveyed as potential substrates for *Leuconostoc mesenteroides*, PRL L33. A significant amount of 12 of 43 sugars, none of 10 sugar alcohols, and two of 13 sugar acids were utilized under aerobic or anaerobic conditions. In addition, acid was produced from a number of the sugars that were not metabolized appreciably. Although the organism has a limited substrate specificity, no essential configurational pattern was observed. Carbon balance studies with K-D-gluconate and K-2-keto-D-gluconate showed that the metabolic products were equimolar amounts of carbon dioxide, lactate, and ethanol plus acetate. Similar products were formed from D-fructose but in addition D-mannitol was produced.

Introduction

Leuconostoc mesenteroides was shown by DeMoss *et al.* to have a metabolic pattern for D-glucose different from the known pathways of carbohydrate metabolism (7). The products from the metabolism of 1 mole of glucose were 1 mole of carbon dioxide, 1 mole of lactate, and 1 mole of ethanol. Because each carbon of the glucose molecule may be easily separated and identified in the products, and there is little, if any, mixing, as shown by experiments with radioactive substrate (6), the organism has been widely used in the degradation of labeled glucose (2, 4). A similar specific degradative pattern was found for labeled D-xylose (1) and D-xylulose (19) and the organism has proved useful in the degradation of these five carbon sugars (2, 19). Thus, besides providing evidence of a new metabolic pathway, this organism is of value in the biological degradation of labeled sugars. In the hope of extending the usefulness of this organism in the biological degradation of carbohydrates a survey was made of a representative number of potential substrates including sugars, polysaccharides, sugar acids, and sugar alcohols. Carbon balances were determined on substrates that were utilized by this organism and on which no previous data were found.

Materials and Methods

Organism

The culture of *Leuconostoc mesenteroides*, PRL L33 (labeled strain 39), was obtained through the courtesy of Dr. R. D. DeMoss now of the University of Illinois. The organism was carried on a broth or agar medium suggested by DeMoss as previously described (1).

[1]*Manuscript received September 4, 1956.*
Contribution from the National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan. Issued as Paper No. 233 on the Uses of Plant Products and as N.R.C. No. 4137. The material was presented at the Sixth Annual Meeting of the Canadian Society of Microbiologists held in Ste. Anne de Bellevue, Que., June 21–23, 1956.

Substrates

The substrates, unless otherwise described, were obtained from commercial sources. K-2-keto-D-gluconate was recovered and purified from a fermentation solution of *Xanthomonas uredovorus*, PRL F21, grown on a glucose-nutrient medium. L-Erythulose was obtained by oxidative fermentation of *meso*-erythritol by *Acetobacter suboxydans*, PRL G3. D-Xylulose was prepared by oxidative fermentation of D-arabitol by *Acetobacter suboxydans*, PRL G3. D-Ribulose was prepared by alkali isomerization of D-arabinose (9). 2-Deoxy-D-glucose was prepared by the method of Overend *et al.* (15) and 6-deoxy-6-fluoro-D-glucose by a modification of the method of Helferich and Vock (10).

The following compounds were gifts from members of this laboratory. D-Arabitol was isolated by Dr. J. F. T. Spencer and Dr. J. M. Roxburgh from the culture filtrates of a strain of osmophilic yeast. D-Threose and D-erythrose were synthesized by Dr. A. S. Perlin. D-Lyxose, D-allose, D-altrose, D-gulose, L-glucose, the heptoses and heptuloses, L-glucitol, L-iditol, Ca-L-arabonate, Ca-D-arabonate, Cd-D-galactonate, L-galactono-γ-lactone, D-talonic acid, Cd-D-gulonate, α-D-galacturonic acid, L-mannonic lactone, D-mannonic lactone, and Ca-L-gluconate were obtained from Dr. A. C. Neish.

Survey

In the survey, 1.0 ml. of medium containing 0.5% tryptone (Difco), 0.5% yeast extract (Difco), 0.001 ml. of a 1.6% alcoholic solution of bromcresol purple, and 1.0% of the test substrate was sterilized, usually by autoclaving, but some labile substrates were sterilized by filtration. After inoculation, the tubes were incubated at 30° C. for one week without agitation under aerobic or anaerobic conditions (anaerobiosis was obtained by placing the tubes in a vacuum desiccator which was evacuated and filled with oxygen-free nitrogen several times). After incubation the pH was recorded; the fermentation solutions were freeze-dried and made to volume with distilled water. The amount of substrate utilized was determined by analysis for sugars with Somogyi (18) and Nelson (14) reagents; the sugar acids and alcohols were estimated by measuring the amount of formaldehyde formed after oxidation with periodate (12); and 2-deoxy-D-glucose was estimated with diphenylamine reagent as described by DeMoss and Happel (8). These were compared to controls and the per cent substrate utilized was calculated. All samples were checked by ascending paper chromatography using a single phase *n*-butanol–pyridine–water solvent (50 : 20 : 18) on Whatman No. 54 paper run for 16 hr. at 30° C. in duplicate. One sample was developed with alkaline silver nitrate (20) and the other with aniline malonate (11).

Carbon Balances

The fermentations were carried out in modified Erlenmeyer flasks (13) on a medium containing 1% yeast extract, 1% tryptone, 0.5% K_2HPO_4, 2% salt mixture (0.8% $MgSO_4 \cdot 7H_2O$, 0.04% $FeSO_4 \cdot 7H_2O$, 0.04% NaCl, t0.19% $MnCl_2 \cdot 4H_2O$, and 0.5% concentrated HCl), a weighed amoun

(ca. 1%) of $CaCO_3$, and the substrate (ca. 500 mgm.). The various constituents were sterilized separately and combined at the time of inoculation. The flasks were incubated on a rotary shaker at 30° C. for about a week under a nitrogen atmosphere.

The carbon dioxide was collected in sodium hydroxide by passing CO_2-free air through the culture liquor after acidification (12). The fermentation solutions were then clarified with zinc hydroxide (12) and made to volume. The ethanol was distilled from an aliquot of the clarified fermentation solution and the acids, after extraction with ether from the residue, were separated on a silica gel column (12). The residual substrate and mannitol were determined on an aliquot of the clarified fermentation solution; fructose was measured with the Somogyi (18) and Nelson (14) reagents; gluconate and mannitol by estimating formaldehyde formed on periodate oxidation (12); and 2-ketogluconate by a modification of the arsenomolybdate reagent described by Perlman (17).

Results and Discussion

Substrate Survey

Of the 66 substrates tested only 12 sugars and two sugar acids were metabolized to an appreciable extent (Table I) demonstrating limited specificity in the range of carbohydrates utilized by this organism. These data are for anaerobic metabolism but the same substrates were utilized aerobically. The presence of oxygen appeared to have little effect on the rate or pattern of carbohydrate utilization by this organism. No sugar alcohols were utilized. Unexpectedly gluconate and 2-ketogluconate were easily and rapidly metabolized.

In Bergey's Manual (5) *Leuconostoc mesenteroides* is reported to produce acid from some of the substrates which were not utilized to any extent by this organism. However, as indicated in Table I an acid reaction was found with a number of substrates that were not utilized. Note that an acid reaction was found with all those substrates that were metabolized. Many of the lactones gave a mild acid reaction even in the controls because of slow hydrolysis of the lactone ring and these doubtful results were not recorded. Although in Bergey's Manual (5) acid is reported from D-mannitol, none was found with this culture. A recent report by Barzotti and McCleskey (3) describes some types of *L. mesenteroides* that produce mannitol and some that do not; possibly the latter can utilize mannitol producing acids, which may explain this anomaly. This culture, when actively growing, gave visible gas production on many of the substrates; a result of rapid production of carbon dioxide as no other gaseous product has been reported (2, 6, see Table II).

An examination of the structural formulae of the compounds utilized by the organism failed to disclose any configurational pattern that was common to all or even a majority of these substrates. Obviously there is no easy explanation of the specificity of this organism towards the various carbohydrates.

TABLE I

UTILIZATION OF VARIOUS CARBOHYDRATE COMPOUNDS BY *Leuconostoc mesenteroides*, L33

(The per cent substrate utilized anaerobically is given if a significant amount disappeared. "Tr." indicates slight but consistent metabolism of the substrate. "*" indicates that an acid reaction was found although no appreciable amount of substrate was utilized.)

Substrates utilized	Substrates not utilized
Sugars	
D-Threose (69), L-erythulose (70)	D-Erythrose
D-Ribose (54), L-arabinose (47), D-xylose (56)	D-Arabinose, D-lyxose*
D-Xylulose (100)	D-Ribulose*
D-Glucose (100), D-mannose (44)	D-Galactose* tr., D-allose, D-altrose, D-gulose, L-glucose
D-Fructose (84)	L-Sorbose D-Gluco-D-guloheptose, L-gala-D-glucoheptose, sedoheptulose,* D-glucoheptulose
β-Methyl-D-glucoside (38)	α-Methyl-D-glucoside* tr., 3-methyl-D-glucose, 2-deoxy-D-glucose, 6-deoxy-6-fluoro-D-glucose
Sucrose (100), turanose (80)	Maltose,* lactose,* melibiose,* cellobiose,* trehalose,* raffinose,* melezitose, rhamnose, esculin, D-glucosamine, dextrin,* starch, inulin
Sugar alcohols	Glycerol, *i*-erythritol, D-arabitol, adonitol, dulcitol tr., L-glucitol, D-mannitol, L-sorbitol, L-iditol, *i*-inositol
Sugar acids	
K-D-gluconate (37)	Ca-D-arabonate, Ca-L-arabonate, Ca-L-gluconate, D-mannonic lactone, L-mannonic lactone, Cd-D-galactonate, L-galactono-γ-lactone, D-talonic acid, Cd-D-gulonate
K-2-keto-D-gluconate (40)	D-Glucuronolactone, α-D-galacturonic acid

Carbon Balances

Previously, carbon balances for this strain of *L. mesenteroides* were reported for D-glucose (6) and for the pentoses, L-arabinose (16), D-xylose (1), and D-xylulose (19). In Table II, carbon balance data are given for D-fructose, K-D-gluconate, and K-2-keto-D-gluconate. The fermentations were complete, carbon recovery was very good, and the oxidation–reduction balances satisfactory, showing that all the major products were found and in the correct amounts.

D-Gluconate and K-2-keto-D-gluconate were metabolized to give products similar to those formed from D-glucose except that both acetate and ethanol are formed from gluconate and only acetate is formed when 2-ketogluconate is the substrate. These results are to be expected, since both of the acids are more highly oxidized than glucose, resulting in the formation of more highly oxidized products. From 1 mole of either substrate utilized, 1 mole of carbon dioxide, 1 mole of lactate, and 1 mole of acetate plus ethanol were formed.

TABLE II

ANAEROBIC DISSIMILATION OF FRUCTOSE, GLUCONIC ACID, AND 2-KETOGLUCONIC ACID BY *Leuconostoc mesenteroides*

(Conditions: about 500 mgm. of substrate were fermented under anaerobic conditions for seven days at 30° C. The pH was controlled by the addition of a known amount of calcium carbonate.)

	mM. product per 100 mM. substrate metabolized		
	D-Fructose	K-D-Gluconate	K-2-keto-D gluconate
CO_2	67	96	98
Ethanol	47.3	41	Trace
Acetate	15.7	52.5	101
Lactate	63.5	98	99
Succinate	Trace	Trace	0
Mannitol	33	—	—
Substrate fermented, %	99.1	99	97.5
Carbon accounted for, %	97	96	99
O/R balance	1.05	1.05	0.98

Fructose, also, was fermented to give products similar to those obtained from glucose except that one-third of the fructose was reduced to mannitol, which was isolated and characterized as the hexaacetate (m.p. 127° C., mixed m.p. 126° C.; $([\alpha]_D^{\text{H}} = +29.9°$ (*c*, 2.04, chloroform)). Since fructose is reduced to mannitol, the other products must be more oxidized to maintain the oxidation–reduction ratio; thus acetate is formed, as ethanol and acetate are apparently in equilibrium. Equimolar amounts of carbon dioxide, lactate, and ethanol plus acetate were found. According to the recent report of Barzotti and McCleskey (3) the amount of mannitol formed could have been increased if the fermentation solution had a lower initial pH. However, only the metabolic pathway of *L. mesenteroides*, PRL L33 (strain 39), has been investigated and it is not known if the other types of this organism such as described by Barzotti and McCleskey (3) metabolize fructose or other sugars in the same manner.

Although the same products are formed from these sugar acids as from glucose, the use of labeled substrates is necessary before deciding whether the mechanism of metabolism of these compounds is similar to that of glucose. If the carbon atom relationships between the substrates and products are similar, enzymatic studies will be necessary to discover the over-all relationship in the metabolism of these compounds. It is hoped to continue this study when labeled substrates are available. If the carbon atom relationships between substrate and products are the same the organism will be useful as a tool in the biological degradation of these labeled substrates in addition to glucose and the pentoses.

The metabolic pathways of the utilization of the tetroses by this culture are unknown. Studies are in progress in an attempt to find the products of metabolism and perhaps obtain an insight into the mechanism involved in degrading these rather rare and labile sugars.

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PROTEINASE BIOSYNTHESIS BY STREPTOCOCCUS LIQUEFACIENS

I. THE EFFECT OF CARBON AND NITROGEN SOURCES, pH, AND INHIBITORS[1]

By Robert Rabin and Leonard N. Zimmerman

Abstract

Some nutritive aspects of proteinase biosynthesis by non-proliferating cells of *Streptococcus liquefaciens*, strain 31, were investigated by substituting constituents in a basal medium containing casein, lactose, purines, pyrimidines, vitamins, and salts. The casein of the medium could be replaced by a mixture of 12 "essential" amino acids (glutamic acid, histidine, valine, serine, methionine, leucine, isoleucine, arginine, cystine, lysine, tryptophane, and threonine), thus demonstrating that proteinase synthesis can occur in a medium devoid of protein. Proteinase biosynthesis appeared to depend upon an inordinately high concentration of arginine, required a fermentable carbohydrate, and occurred optimally at pH 6.3. Sodium fluoride and iodoacetate did not inhibit the proteinase activity but radically curbed its synthesis.

Introduction

Recently Grutter and Zimmerman (8) isolated an extracellular proteolytic enzyme from *Streptococcus zymogenes* that seemed to be similar to if not identical with (6) the proteolytic enzyme isolated from the closely related species *Streptococcus liquefaciens* by Dudani and Nelson (3). Preliminary studies had indicated that this enzyme was adaptive in nature for both organisms (3, 7). Because of the consistently greater activity exhibited by the latter species, it was used in the present work to determine some of the prerequisites of proteinase biosynthesis. Such a study might prove fruitful in that most work of this nature has been carried out on the enzymes associated with carbohydrate metabolism or with organisms whose nutritive requirements are far less complex than those of the streptococci (9, 13, 15, 18).

Materials and Methods

Organism Used and Conditions of Culture

Streptococcus liquefaciens, strain 31, obtained from the Department of Bacteriology, Cornell University, was used throughout this work. Stock cultures were maintained at −17° C. in litmus milk. Cultures were prepared for enzyme biosynthesis by first growing a 10% inoculum for six hours in A-C broth, which consisted of the following ingredients: Sheffield N-Z Case, 1.0 gm.; yeast extract, 1.0 gm.; K_2HPO_4, 0.2 gm.; glucose, 0.1 gm.; and distilled water to 100 ml. The pH was adjusted to 7.0.

[1]*Manuscript received July 23, 1956.*
Contribution from the Department of Bacteriology, The Pennsylvania State University, University Park, Pennsylvania. This report is from a dissertation submitted by Robert Rabin in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of The Pennsylvania State University. Authorized for publication on April 16, 1956, as paper No. 2056 in the Journal Series of the Pennsylvania Agricultural Experiment Station. Part of this work was presented at the 55th General Meeting of the Society of American Bacteriologists at New York, N.Y., May 8–12, 1955.

Cells were centrifuged, washed once, resuspended in distilled water, and inoculated into a casein semisynthetic medium (CSM) of the following composition: vitamin-free casein, 0.2 gm.; KH_2PO_4, 1.0 gm.; lactose, 0.2 gm.; NaCl, 0.2 gm.; $MgSO_4 \cdot 7H_2O$, 8 mgm.; $CaCl_2$, 1.5 mgm.; $FeSO_4 \cdot 7H_2O$, 0.4 mgm.; $MnSO_4 \cdot H_2O$, 0.16 mgm.; $CoCl_2$, 0.12 mgm.; adenine, 0.5 mgm.; guanine, 0.5 mgm.; uracil, 0.5 mgm.; niacin, 0.1 mgm.; pyridoxine . HCl, 0.1 mgm.; riboflavin, 0.1 mgm.; calcium pantothenate, 0.1 mgm.; folic acid, 1.0 μgm.; biotin, 0.1 μgm.; and distilled water to 100 ml. The final pH was adjusted to 7.0.

After incubation for 17–18 hr., the cells were centrifuged in the cold at 15,300 $\times$ *g* for three minutes and washed twice with an amount of distilled water corresponding to 10% of the final growth medium. The bacteria were then suspended in a volume of distilled water that was equal to 4% of the final growth medium.

Amino acids were substituted for the casein in the CSM for those experiments requiring cells grown on a completely synthetic medium (SM). The amount of each amino acid in the medium was based on its approximate concentration in 0.2% casein (2) with appropriate compensations made when "DL" forms were used. The amino acids in 100 ml. of SM are as follows: L-histidine . HCl . H_2O, 6.0 mgm.; L-lysine . 2HCl, 16.8 mgm.; L-tyrosine, 12.0 mgm.; L-tryptophane, 3.0 mgm.; DL-methionine, 13.6 mgm.; DL-serine, 24.8 mgm.; DL-threonine, 16.4 mgm.; L-leucine, 20.6 mgm.; DL-isoleucine, 24.8 mgm.; DL-valine, 27.2 mgm.; L-glutamic acid, 46.6 mgm.; L-arginine . HCl, 7.6 mgm.; DL-alanine, 14.8 mgm.; DL-phenylalanine, 20.0 mgm.; L-proline, 21.2 mgm.; L-cystine, 0.7 mgm.; DL-aspartic acid, 28.4 mgm.; glycine, 4.0 mgm. Stock cultures that had been carried through five consecutive transfers on SM, and frozen in the same medium, were used in preparing inocula for these experiments. These cultures were prepared for enzyme biosynthesis in the manner previously described for the other cells, except that the SM was used throughout.

Conditions Employed for Enzyme Biosynthesis

Five milliliters of cell suspension was added to 45 ml. of media in a 125 ml. flask and incubated, usually for five hours in a water bath. Essentially a modified CSM (MCSM) was used for biosynthesis; the K_2HPO_4 concentration and pH adjustment were altered to 0.110 *M* and 6.5, respectively. Other substitutions were also made in studying the nutrition of biosynthesis, but they will be mentioned in the next section.

Normally the cell concentration in the medium was approximately 2×10^9/ml. Under no circumstances did cell proliferation occur during these incubation periods. This fact was ascertained by plate counts on A-C agar (1.5% agar added to A-C broth) and by turbidity measurements with the Evelyn photoelectric colorimeter.

Assay of Enzymatic Activity

Biosynthesis was usually concluded by immersing the cell suspension in an ice bath. The material was then centrifuged in the cold at 15,300 × *g* for three minutes and the supernatant fluid was assayed for proteinase activity by measuring the tyrosine solubilized according to the methods described in previous papers (1,8).

Alternate procedures were necessary in two experiments, to obtain an estimate of proteinase activity. The determination of adequate carbon and/or energy substitutes for lactose was measured directly in the medium by analyzing for the solubilized tyrosine. Where optimum pH for enzyme biosynthesis was ascertained, it was necessary to prepare an ammonium sulphate fraction for each pH value tested, according to the method described by Grutter and Zimmerman (8).

Use of Inhibitors

The various inhibitors were prepared at 1 *M* concentrations. Their effect on enzyme activity was tested by adding 0.1 ml. of inhibitor solution to each tube at the start of the assay, and their effect on proteinase biosynthesis was obtained after 0.5 ml. was added to culture flasks prior to inoculation. Water replaced inhibitor solution in the controls.

Temperature Control

The temperature of incubation for growth, enzyme biosynthesis, and enzyme assay was always 37° C.

Reproducibility of Results

Each set of data as reported represents the results of a typical individual experiment. Although a great deal of variation with respect to the over-all level of proteinase synthesis occurred from time to time, the relative percentages for the various materials tested within any single series of experiments remained fairly constant (never greater than 10%).

Experimental Results

Conditions Necessary for Enzyme Biosynthesis

1. *Optimum pH*

Dudani and Nelson (3) had demonstrated that the optimum pH for proteinase activity of *Streptococcus liquefaciens* was 7.4. Because proteinase could be produced in a medium in which the pH did not go above 7.0, it was decided to determine the optimum pH for biosynthesis. Accordingly, flasks of MCSM of various pH's were inoculated and the pH in each flask was readjusted to its original reaction at half hour intervals with 0.5 *N* NaOH. Except for the flask initially adjusted to pH 5.40, pH variation during incubation never exceeded 0.1 units in any of the other flasks. Fig. 1 shows that the optimum pH for enzyme biosynthesis is 6.3, with as much as a 50% decrease in activity at pH 6.5.

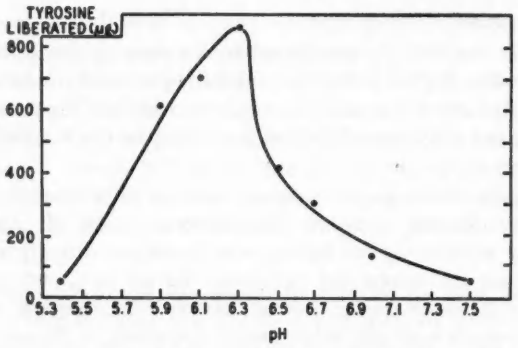

FIG. 1. Optimum pH of enzyme formation.

2. *Carbon and/or Energy Substitutes for Lactose*

Various compounds were checked for their ability to replace lactose during enzyme biosynthesis. Some flasks of MCSM were brought to pH 6.2 for these experiments, in addition to the usual adjustment to pH 6.5. This was done to provide a more favorable pH for biosynthesis in those flasks containing compounds (acetate, lactate, ATP, etc.) that could not be fermented. Only the fermentable carbohydrates glucose and galactose were capable of serving as adequate substitutes for lactose (Table I); adenosine triphosphate (ATP) had little effect.

3. *Casein Substitutes*

The possibility of replacing casein in the MCSM was investigated by devising several media in which the casein was replaced by the following components: 0.2% gelatin; an amino acid mixture based on concentrations described for the SM; the amino acid mixture plus 0.2% gelatin; the amino acid mixture plus 0.0032 *M* glycylglycine; the amino acid mixture plus 0.0032 *M* glutathione; the amino acid mixture plus 0.0032 *M* arginine as α-benzoyl-L-arginine amide; the amino acid mixture plus 0.0016 *M*

TABLE I

ENZYME FORMATION IN THE PRESENCE OF ATP AND/OR VARIOUS CARBON SOURCES

pH 6.5	μgm. tyrosine solubilized in 5 hr.	pH 6.2	μgm. tyrosine solubilized in 5 hr.
Lactose	270	Lactose + ATP	245
Lactose + ATP	291	α-Ketoglutaric acid + ATP	55
Glucose	203	Sodium lactate + ATP	31
Galactose	226	Sodium pyruvate + ATP	15
Citric acid + ATP	28	Sodium acetate + ATP	31
Sodium lactate + ATP	59	Glutamic acid + ATP	17
Sodium pyruvate + ATP	74	Glycine + ATP	36
Succinic acid + ATP	37	ATP	46

NOTE: *ATP is present at a concentration of 100 μgm. per ml.; the carbon sources are at the 0.2% level.*

carbobenzoxy-L-glutamyl-L-tyrosine. (Molarity is reported as a final concentration throughout this paper.) The peptides could not induce biosynthesis; only in the amino acid mixture plus gelatin was there demonstrable activity.

4. *Amino Acid Requirements in the Presence of Gelatin*

Several experiments were designed to find which of the 18 amino acids were necessary in the presence of 0.2% gelatin for proteinase biosynthesis. Preliminary tests revealed that optimal activity was obtained when arginine, valine, and serine were used at a 0.0032 *M* concentration instead of the level previously mentioned.

With the single removal of each of the 18 amino acids (Table II) it can be seen that aspartic acid exerts an inhibitory influence. In addition, if 90% activity is taken as a critical level, then there are nine amino acids (aspartic acid, lysine, phenylalanine, alanine, glycine, tyrosine, serine, threonine, and proline) that might be considered non-essential. A medium containing the remaining nine essential amino acids plus gelatin, however, was able to yield only 61% of the anticipated activity.

TABLE II

INFLUENCE OF SINGLE OMISSION OF AMINO ACIDS ON ENZYME FORMATION

	μgm. tyrosine solubilized in 60 min.	% activity
Experiment No. 83		
All 18 amino acids	466	100
minus arginine	180	39
minus tryptophane	9	2
minus glutamic acid	0	0
minus valine	99	21
minus leucine	332	71
minus aspartic acid	560	120
minus methionine	316	68
Experiment No. 84		
All 18 amino acids	476	100
minus histidine	26	5
minus lysine	433	91
minus phenylalanine	488	103
minus alanine	479	101
minus glycine	505	106
minus isoleucine	207	44
minus cystine	395	83
Experiment No. 85		
All 18 amino acids	477	100
minus tyrosine	454	95
minus serine	444	93
minus threonine	463	97
minus proline	477	100

NOTE: *The gelatin content of each flask is 0.2%. Arginine, valine, and serine when used are at the 0.0032 M level.*

TABLE III

INFLUENCE OF SINGLE OMISSION OF THE 11 ESSENTIAL AMINO ACIDS ON ENZYME FORMATION

	μgm. tyrosine solubilized in 60 min.	% activity
Experiment No. 90		
All 11 amino acids	504	100
minus glutamic acid	57	11
minus histidine	77	15
minus valine	155	34
minus serine	372	74
plus 7 non-essential	548	108
Experiment No. 91		
All 11 amino acids	466	100
minus methionine	356	76
minus leucine	348	75
minus isoleucine	303	65
minus arginine	51	11
minus cystine	354	76
minus lysine	244	52
minus tryptophane	12	3

NOTE: *The gelatin content of each flask is 0.2%. Arginine, valine, and serine when used are at the 0.0032 M level. The 11 amino acids include all of the listed succeeding individual amino acids. The non-essential amino acids are glycine, aspartic acid, proline, alanine, phenylalanine, threonine, and tyrosine.*

TABLE IV

EFFECT OF AMINO ACILS ON PROTEINASE BIOSYNTHESIS IN THE PRESENCE AND ABSENCE OF GELATIN

Flask No.	Arginine		Serine		Valine		Threonine		0.2% gelatin	μgm. tyrosine solubilized in 60 min.
	High	Normal	High	Normal	High	Normal	High	Normal		
1	+		+		+				+	518
2	+		+		+				−	287
3		+		+		+			−	18
4	+		+		+		+		−	664
5	+		+		+		+		+	643
6	+			+		+		+	+	575
7	+			+		+		+	−	584
8		+		+		+	+		−	23

NOTE: *All flasks contain the 11 essential amino acids listed in Table III. Those amino acids listed as high are present at the 0.0032 M level; those amino acids listed as normal, and all other amino acids in the flasks, occur at concentrations described in the text.*

To avoid the complications accruing from single omission, the experimental design was altered so that amino acid removal was cumulative, rather than singular. For example glycine was left out of flask "*a*" and if found to be non-essential, it was then dropped from flask "*b*" along with a new amino acid that was to be tested. In this manner, seven amino acids (glycine, aspartic acid, proline, alanine, phenylalanine, threonine, and tyrosine) were found to be non-essential (Table III). The omission of any single amino acid from the pool of the 11 essential ones resulted in enzyme losses of approximately 24% or more. Use of all 18 amino acids increased activity by only 8% as compared to the activity when the 11 essential amino acids were used.

5. *Amino Acid Requirements in the Absence of Gelatin*

The possibility of obtaining proteinase production in the absence of casein or gelatin was reinvestigated. Table IV shows that enzyme synthesis can occur, although at a reduced level, in the presence of the 11 essential amino acids, and in the absence of gelatin (see flasks 1 and 2). As was previously stated, the flask 3 data illustrate that when arginine, valine, and serine are present at levels approximating their concentration in 0.2% casein, activity is negligible. Of special interest is the medium (from this point on referred to as MSM) in flask 4 demonstrating the restoration of full activity in the absence of gelatin when threonine, at a high concentration, is added to the other 11 amino acids. Flask 5 shows that in the presence of gelatin, threonine has no effect. The importance of the high arginine concentration in maintaining top level activity is emphasized by flasks 6, 7, and 8. Flask 8 also indicates the inability of only a high threonine concentration to sustain optimum proteinase biosynthesis.

6. *Comparative Enzyme Biosynthesis*

Biosynthetic activity of bacteria grown on the CSM and SM was compared by observing the rate of proteinase production when equal cell concentrations were inoculated into the MCSM and MSM. Aliquots were withdrawn at two hour intervals and analyzed. Fig. 2 shows that the highest levels of enzyme biosynthesis occur in those flasks containing MSM in which there are readily assimilable supplies of amino acids. By the same token, SM-grown cells seem to produce more enzyme than CSM-grown cells when both are inoculated into the same types of media.

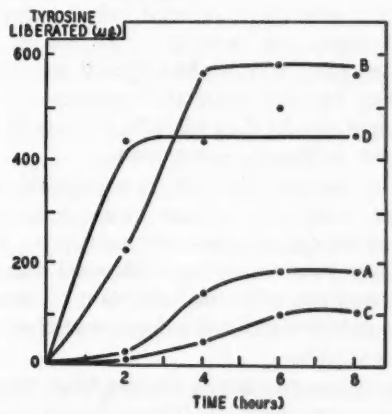

FIG. 2. Rates of proteinase biosynthesis. A—cells harvested from the SM and incubated in the MCSM. B—cells harvested from the SM and incubated in the MSM. C—cells harvested from the CSM and incubated in the MCSM. D—cells harvested from the CSM and incubated in the MSM.

TABLE V

EFFECT OF INHIBITORS

	Proteinase activity		Proteinase biosynthesis	
	μgm. tyrosine solubilized in 60 min.	% inhibition	μgm. tyrosine solubilized in 60 min.	% inhibition
NaF	750	0	100	92
IAc	670	10	95	92
$NaAsO_2$	695	7	948	24
H_2O	745	—	1248	—

Inhibition of Enzyme Biosynthesis

It was shown (Table V) that sodium fluoride (NaF), sodium arsenite ($NaAsO_2$), and iodoacetate (IAc) do not markedly inhibit the proteinase activity. Enzyme biosynthesis, however, is curtailed drastically in the presence of NaF and IAc.

Discussion

Proteinase biosynthesis by *Streptococcus liquefaciens*, strain 31, apparently requires rather specialized conditions. Although the optimum pH for proteinase activity is pH 7.4, the optimum pH for biosynthesis of proteinase is pH 6.3. It is significant that biosynthesis occurs only within narrow pH limits.

Presumably proteinase biosynthesis requires energy as well as a carbon source. ATP and/or various non-fermentable carbon sources could not meet this requirement, which was satisfied only by the fermentable sugars tested (glucose, galactose, and lactose). The use of whole cells in the experimental procedure may have worked against the successful substitution for these sugars. For instance, cellular impermeability to the substances tested, or degradation of the ATP by phosphatase, could account for the lack of stimulation by these alternative compounds.

The inhibitory effect of IAc and NaF re-emphasizes the importance of a source of carbon and energy for enzyme biosynthesis; both inhibitors are known to interfere with carbohydrate fermentation (12). This does not preclude the possibility, however, of an additional site of inhibition. Any enzyme(s) directly concerned with the formation of the proteinase molecule itself could have been inactivated if it contained a sulphydryl group or required a magnesium ion as a cofactor.

The amino acid requirements during protein biosynthesis are of particular interest. In view of the fact that Gale (4) and Taylor (17) have demonstrated the presence of stored amino acids in streptococci, it would not be expected that biosynthesis could proceed only in the presence of added amino acids. Whereas Taylor (17) showed that stored glutamic acid is relatively high among

the streptococci studied, Table II indicates that if a comparable level of this amino acid is present in *Streptococcus liquefaciens*, it is apparently not available for detectable synthesis.

That amino acids are required is partially in keeping with the data of Gale and Folkes (5), who found that free amino acids were necessary for high level, but not low level, glutamic acid incorporation by *Staphylococcus aureus*, even though this organism was also capable of amino acid storage. Halvorson and Spiegelman (10, 11) have obtained adequate levels of enzyme biosynthesis from the yeast, *Saccharomyces cerevisiae*, without any outside source of amino acids. Since a comparison of data (10, 17) show that the stored amino acids tested were similar in concentration for both yeast and streptococci, it is difficult to explain why no, or so little, enzyme is synthesized by our organism upon the omission of certain amino acids. Although it is possible that the organism did not build up an adequate amino acid pool during growth, it is interesting to note that the amino acid that is present during growth in the highest concentration (in casein) is the amino acid that brings synthesis to a complete halt when it is omitted (see data on glutamic acid in Table II).

It is not known, at this time, why higher concentrations of free arginine rather than bound arginine (as in casein) are required for good proteinase synthesis.

The complications arising from using single amino acid omission to determine the essential amino acids necessary for biosynthesis is not surprising, since other investigators (16) have had similar experiences with respect to the nutritive requirements for growth. The basic cause of such complications may arise from interrelationships among the amino acids by way of the transaminating enzymes.

In general, the amino acid growth requirements for the enterococci (14) are quite similar to those required for proteinase biosynthesis by *S. liquefaciens*. With the exception of tyrosine and alanine, which are necessary for optimum growth but not biosynthesis, and cystine, which is necessary for optimum biosynthesis but not growth, the amino acid requirements are alike. Nevertheless, this disparity in amino acid content between the medium of Niven and Sherman (14) and the MSM is enough to prevent growth. An increased cell population could not be demonstrated even when, in a special experiment designed to approach the test conditions employed by Niven and Sherman, sodium thioglycollate was added to the medium and the mass seeding for biosynthetic purposes was replaced by a loop inoculum per 10 ml. It would appear, then, that amino acid requirements for biosynthesis of any particular cellular component are not necessarily identical with those required for growth.

The data contained in Table IV and Fig. 2 show that the formation of proteinase in the presence of readily assimilable amino acids is not dependent on an inducing agent. It might be argued on the basis of data from Table IV (flask 7) that arginine as a product of protein breakdown, and when present at a high concentration, acts as an inducer through the process of simultaneous

adaptation. A more rational approach, perhaps, would be to think of enzyme biosynthesis under these circumstances as being analogous to nitratase formation by *Escherichia coli*. Wainwright (19) has reported that the adaptive enzyme nitratase is produced by this organism in the absence of the nitrate inducer if a high concentration of amino acids is present.

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NOTES

INFECTION BY STREPTOMYCES SCABIES ON DETACHED POTATO TUBERS[1]

By C. H. Lawrence[2]

The common scab disease of potatoes, caused by *Streptomyces scabies* (Thaxt.) Waksman and Henrici, has been reported to develop only on actively growing tubers that are attached to the parent plant (2, 3, 4, 5). However, we have found that, under certain conditions, this disease will develop on detached potato tubers.

Axillary tubers were produced on cuttings from Green Mountain plants by a method similar to that of Busch and Gilpatrick (1). These tubers, which formed in two to four weeks, were 5 to 18 mm. in diameter. Tubers 5 to 40 mm. in diameter from Green Mountain plants grown in pots in the greenhouse were also used. All tubers were carefully harvested to avoid wounding, washed in running water, and immediately inoculated. Tubers up to approximately 18 mm. in diameter were inoculated either by being revolved over the surface growth of a heavy sporulating culture or by being dipped in a mycelium–spore suspension of the test organism. The larger tubers were inoculated by rubbing the surface with a piece of cotton wool dipped in the spore suspension. Three pathogenic and three non-pathogenic cultures were tested. After inoculation the tubers were placed in Petri dishes and covered with sterile fine-grade moist vermiculite. The dishes were then covered and incubated at 28° C.

On the smaller tubers, inoculated with the three parasitic cultures, an initial infection of the lenticels appeared after two days and eruptive scab lesions after a week's incubation. Fewer lesions occurred on the larger tubers and these took longer to develop. Microscopic examination of the scab lesions showed numerous strands of fine mycelium. Isolations from the lesions gave cultures morphologically similar to those used for the inoculations. Tubers inoculated with the non-parasitic cultures failed to show any symptoms after 18 days.

The infection of detached tubers may prove useful in studies of the host–parasite relationship and as a relatively rapid means for testing the pathogenicity of isolates of *S. scabies*.

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RECEIVED AUGUST 15, 1956.
BOTANY AND PLANT PATHOLOGY DIVISION,
CANADA DEPARTMENT OF AGRICULTURE,
OTTAWA, ONTARIO.

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